Neuropharmacological assessment of *Desmodium gangeticum* in

Partial sciatic nerve ligation in rat model: Role of inflammatory mediators

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CERTIFICATE

This is to certify that the dissertation work entitled "**Neuropharmacological assessment of *Desmodium gangeticum* in Partial sciatic nerve ligation in rat model: Role of inflammatory mediators"** submitted by University **Reg. No.261525907** is a bonafide work carried out by the candidate under the guidance of Dr. M. Ramanathan, M. Pharm., PhD., and submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2015-2016.

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DECLARATION

I do hereby declare that the dissertation work entitled "**Neuropharmacological assessment of *Desmodium gangeticum* in Partial sciatic nerve ligation in rat model: Role of inflammatory mediators"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology, was done by me under the guidance of Dr.M.Ramanathan, M.Pharm., PhD., at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2015-2016**.**

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled "**Neuropharmacological assessment of *Desmodium gangeticum* in Partial sciatic nerve ligation in rat model: Role of inflammatory mediators"** submitted by University **Reg. No.261525907** to the Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology is a bonafide work carried out by the candidate at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore and was evaluated by us during the year 2016-2017.

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Dedicated

To The Almighty

My beloved Mother

&

IN LOVING

MEMORY

OF MY FATHER…

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ABBREVIATIONS

COX	**:**	Cyclooxygenase
CMC	**:**	Carboxy methyl cellulose
CCI	**:**	Chronic constriction injury
CPCSEA	**:**	Committee For The Purpose Of Control And Supervision On Experimental Animals
DG	**:**	*Desmodium gangeticum*
DMEM	**:**	Dulbecco's modified Eagle's medium
DMSO	**:**	Dimethyl sulphoxide
DNA	**:**	Deoxy ribonucleic acid
e NOS	**:**	Endothelial nitric oxide synthase
EDTA	**:**	Ethylene diamine tetra acetic acid
ELISA	**:**	Enzyme linked immunosorbent assay
GPN	**:**	Gabapentin

HPTLC	**:**	High performance thin layer chromatography
i NOS	**:**	Inducible nitric oxide synthase
IC_{50}	**:**	Inhibitory concentration 50
IL	**:**	Interleukin
LOX	**:**	Lipoxygenase
LPS	**:**	Lipopolysaccharide
MTT	**:**	3-(4,5-dimethylthiazol – 2yl) -2,5- diphenyl Tetrazolium bromide
n NOS	**:**	Neuronal nitric oxide synthase
NCCS	**:**	National centre for cell science
NO	**:**	Nitric oxide
NSAIDs	**:**	Non-steroidal anti-inflammatory drugs
PCR	**:**	Polymerase chain reaction
PGs	**:**	Prostaglandin
PSNL	**:**	Partial sciatic nerve ligation

STV **:** Saline: Trypsin: Versene

TNF-α **:** Tumour necrosis factor – alpha

Chapter 1

Introduction

1. INTRODUCTION

Pain is one of the most important problems associated with personal and social affliction worldwide, and it remains an important challenge of modern medicine. The International Association for the Study of Pain defined chronic pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Vallath *et al*., 2013). Unfortunately, some individuals experience pain without an obvious injury or suffer protracted pain that persists for months or years after the initial insult. This painful condition is usually neuropathic in nature and accounts for a large number of patients presenting with chronic, non-malignant pain to clinics. Neuropathic pain is defined as neural damage in the nervous system which leads to pain and loss of function. International association for the study of pain (IASP) has given a new definition for neuropathic pain as "It is initiated or caused by a primary lesion, dysfunction or transitory perturbation of the peripheral or central nervous system" (Loeser and Treede., 2008). It commonly occurred in patients with cancers, virus infections, diabetes, spinal cord injury, peripheral nerve injury and ischemia. Neuropathic pain is characterized by hypersensitivity to nonnoxious (allodynia) and noxious (hyperalgesia) stimulation (Baron *et al*. 2009). Allodynia is mediated via Aβ fibers, and human studies have demonstrated that the blockade of Aβ fibers can abolish allodynia (Torebjork *et al.,* 1992). Hyperalgesia is mediated via sensitized Aδ fibersor C-fibers (Ziegler *et al.,* 1999). In the periphery the injured nerve fibers releases several inflammatory mediators such as bradykinin, serotonin, prostaglandins, adenosine triphosphate (ATP) and protons, which increase the sensitivity and excitability of the nociceptive neurons either directly or by activation of intracellular signaling pathways (Julius and Basbaum, 2001; Ji *et al.,* 2003) . Calcitonin gene-related peptide (CGRP) and substance P induce hyperaemia and swelling. This facilitates the

invasion of immune cells guided by chemokines to the side of injury. Immunecells in turn release proinflammatory mediators like prostaglandins and cytokines, e.g. Interleukin (IL - 1β, IL-6) interferon γ and tumor necrosis factor α (TNFα) (Scholz and Woolf, 2007). These mediators excite neurons and aid in the initiation of inflammation via chemoattraction and vasodilation. Exogenous administration of such agents (e.g., TNF-α, PGE2)has resulted in pain-like behavior (Jin and Gereau, 2006;) Recent studies has shown celecoxib, a COX 2 inhibitor reverted oxaliplatin-induced neuropathic pain by inhibition of PI3K/Akt2 pathway in the mouse dorsal root ganglion (Jiang et al., 2016).As far as we knew, neuropathic pain is developed as a consequence of Neuro inflammatory signaling and central sensitization in the spinal cord (Campbell and Meyer, 2006; Ji and Strichartz, 2004; Tsuda *et al*., 2005). Since psychosocial stress is often endured alongside these conditions (Gold et al., 2005; Scadding and Koltzenburg, 2006; Strang, 1998) and clinical observations suggest that stress increases susceptibility to develop pain and exacerbates existing pain (DeLeo, 2006; Greco *et a*l., 2004; Nicholson and Martelli, 2004; Turner *et al.*, 2002), it is important to understand how stress affects the development and severity of neuropathic pain.

Accumulating evidence demonstrates that currently available treatment for neuropathic pain, such as non-steroid anti-inflammatory drugs, morphine, anti-convulsants and anti-depression drugs, has limited efficiency, and is often accompanied by unfavorable side effects.Natural products are the most consistently successful sources of drug leads. The screening of natural products can provide greater structural diversity than standard synthetic chemistry and offers significant opportunities for finding novel low molecular weight lead compounds. With reference more than 50 percent of FDA-approved drugs are derived from natural products (Daniel et al., 2012).*Desmodium gangeticum* , an Indian medicinal plant has been widely used by many

Ayurvedic and Unani physicians for curing fever, cataract, typhoid, piles, bronchitis, dysentery, asthma and various other inflammatory conditions arising from 'vata' disorder(Subha Rastogi *et al.,* 2011) Flavonoid and alkaloid fractions of *Desmodium gangeticum* were evaluated for anti inflammatory and antioxidant activities in carrageenan-induced inflamed rats with the aim of studying the promising fraction for inhibitory action on ferrous sulphate induced lipid peroxidation, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and total reduced glutathione in liver and spleen homogenates of inflamed rats.(Govindaraj *et al.,* 2007)

So, in this study *Desmodium gangeticum*will be screened for its anti inflammatory and neuroprotective activity in *invitro* and *invivo* models. *Desmodium gangeticum* will be screened priminarily in LPS intoxicated raw 264.7 model for its anti inflammatory activity. The neuroprotective mechanism of action of the *Desmodium gangeticum* has been evaluated in Partial sciatic nerve ligation (PSNL) injury model. *Desmodium gangeticum* will also be screened for their effect in improving pain associated cognitive impairment.

Chapter 2

Literature review

2. LITERATURE REVIEW

The mechanism underlying neuropathic pain is complex yet it is essential for the perception of pain. The onset of neuropathic pain is due to the lesions in the somatosensory system, induced either in the peripheral or the central part of the nervous system. This phenomenon that occurs in both the peripheral and central nerve is thought to cause the persistence of the chronic neuropathic pain (Campbel *et al.,* 2005). Over the years, clinical studies have explored the different bases of neuropathic pains from post-surgical cases to diabetics; specifically, divided physical causes of pain into two types: nociceptive and neuropathic pain.

I. NOCICEPTIVE PAIN

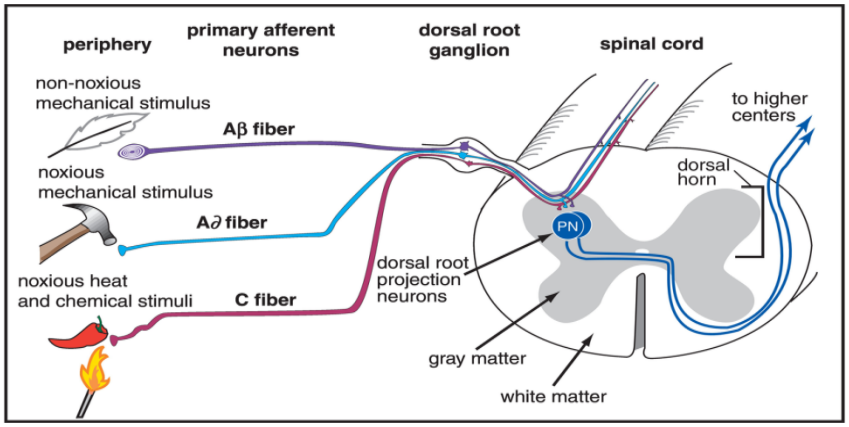
Nociceptors are the nerves which sense and respond to parts of the body which suffer from damage. When activated, they transmit pain signals (via the peripheral nerves as well as the spinal cord) to the brain. The pain is typically well localized, constant, and often with an aching or throbbing quality. Nociceptive pain is mediated by receptors on A-delta and C-fibers which are located in skin, bone, connective tissue, muscle and viscera. These receptors serve a biological useful role at localizing noxious chemical, thermal and mechanical stimuli. Nociceptive pain can be somatic or visceral in nature. Somatic pain tends to be well localized, constant pain that is described as sharp, aching, throbbing, or gnawing (Costigan *et al.,* 2009). Visceral pain, on the other hand, tends to be vague in distribution, paroxysmal in nature and is usually described as deep, aching, squeezing and colicky in nature. Visceral pain is the subtype of nociceptive pain that involves the internal organs (Basbaum *et al.,* 2001) Examples of nociceptive pain include: post-operative pain, pain associated with trauma, and the chronic pain of arthritis. (Richeimer *et al.,* 2010)

II. NEUROPATHIC PAIN

Neuropathic pain, in contrast to nociceptive pain, is described as "burning", "electric", "tingling", and "shooting" in nature. It can be continuous or paroxysmal in presentation. Whereas nociceptive pain is caused by the stimulation of peripheral of A-delta and C-polymodal pain receptors, by algogenic substances (e.g. Histamine bradykinin, substance P, etc.) neuropathic pain is produced by damage to, or pathological changes in the peripheral or central nervous systems(Dworkin *et al.* 2003, Woolf & Mannion 1999)Examples of pathological changes include prolonged peripheral or central neuronal sensitization, central sensitization related damage to nervous system inhibitory functions, and abnormal interactions between the somatic and sympathetic nervous systems. (Clin J Pain *et al.*, 2000) The hallmarks of neuropathic pain are chronic allodynia and hyperalgesia. Allodynia is defined as pain resulting from a stimulus that ordinarily does not elicit a painful response (e.g. Light touch). Hyperalgesia is defined as an increased sensitivity to normally painful stimuli. Primary hyperalgesia, caused by sensitization of C-fibers, occurs immediately within the area of the injury. Secondary hyperalgesia, caused by sensitization of dorsal horn neurons, occurs in the undamaged area surrounding the injury. Examples include post herpetic (or post-shingles) neuralgia, reflex sympathetic dystrophy causalgia (nerve trauma), components of cancer pain, phantom limb pain, entrapment neuropathy (e.g., carpal tunnel syndrome), and peripheral neuropathy (widespread nerve damage). (Richeimer *et al*., 2010)

III.MIXED CATEGORY PAIN - In some conditions the pain appears to be caused by a complex mixture of nociceptive and neuropathic factors. An initial nervous system dysfunction or injury may trigger the neural release of inflammatory mediators and subsequent neurogenic inflammation. For example, migraine headaches probably represent a mixture of neuropathic and

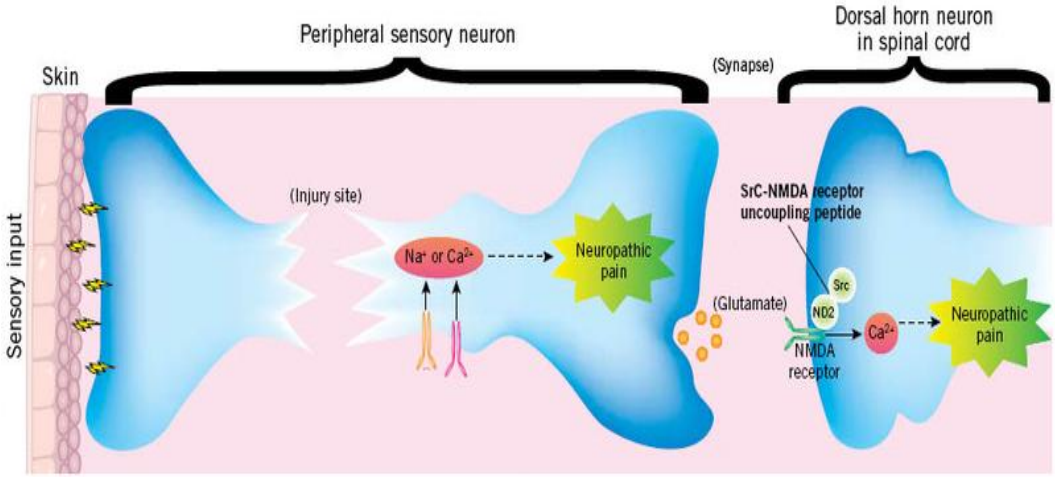
nociceptive pain. Myofascial pain is probably secondary to nociceptive input from the muscles, but the abnormal muscle activity may be the result of neuropathic conditions. And remember, treating the physical, as well as using the mind's ability to heal itself, will optimize the treatment process. Examples of neuropathic pain include: monoradiculopathies, trigeminal neuralgia, post herpetic neuralgia, phantom limb pain, complex regional pain syndromes and the various peripheral neuropathies. (Steven Richeimer *et al.*, 2010)



***Receptor, neurotransmitters, enzymes, channels**:*

Signs of neuropathic pain can be related to the tingling, inching, and burning sensations. The locality of the pain is also important indicators to determine the origin and the sprout of the pain, usually from the peripheral nerves and then extending to the central nerves. However, these sensations of pain can be further analyzed on the molecular basis through signal transduction. Some key players involved during pain signaling may include the release of neurotransmitters and neuropeptides such as glutamate and substance P; receptors such as the AMPA, NMDA, and Glu receptors; the enzymes such as AMP-activated protein kinase activators (Tillu and D.V., Price 2012); and the gatekeepers which include the sodium and calcium channels**. in** addition, brain-derived neurotrophic factors (BDNF), nerve growth factors (NGF), and glia-cell derived neurtrophic factors (GDNF) also influence neuropathic pain. Neurotrophins, which are usually

involved in the development of sensory systems and neuronal plasticity, can mediate and indicate the underlying mechanisms of neuropathic pain. Moreover, a number of other neuropeptides such as endomorphins, dynorphin A, and galanin can also induce nerve stimulation. The modulation and alteration of each player in the signal transduction cascade contribute to the primary afferent hyperexcitability; all in all, leading to the perception of neuropathicpain.(Campbell *et al.*,2006)



Pre/post – synaptic mechanisms

The mechanism of neuropathic pain can be first evaluated in the pre- and post- synaptic regions. In a normal undamaged neuron, Glutamate, which is an excitatory neurotransmitter, is usually restrained by the inhibitory g-protein coupled receptors (i.e. adenosine and GABA-B receptors) However, once an injury has occurred on the site of peripheral nerves, anatomical changes can take place in excitatory synaptic transmission to up regulate the activity of postsynaptic glutamate AMPA receptor-mediated response and the glutamate release. The increased glutamate release leads to the amplification of neuronal firing rate and depolarization resulting in the sensitization of the nerves to produce pain (Zieglgänsberge *et al.,* 2005) The upregulation of

GluR1 receptors in the post-synapse is a well defined marker for the augmentation of glutamate release. The damages to the peripheral sensory neurons consequently affect the central nervous system through further release of glutamate, substance P and brain-derived neuropathic factor (BDNF) in the primary nociceptor afferent at the central terminal. Substance P binds to the neurokinin 1 (NK1) whereas the BDNF binds to the tyrosine kinase receptor (trk) on the post synaptic membrane. The gradual depolarization and phosphorylation of the NMDA glutamate receptor in the post-synaptic surface is followed by the increase in calcium concentration and cAMP subsequently activates the protein kinases C (PKC). Furthermore, the NMDA receptor will also stimulate the production of nitric oxide synthetase to promote other excitatory amino acid and neuropeptide release from the pain fibers. The overall signalling cascade sensitizes the dorsal horn by spontaneous activation and contributes to the modulation of pain. (Tomiyama *et al.,* 2005)

PATHOPHYSIOLOGY

The mechanisms involved in neuropathic pain are complex and involve both peripheral and central pathophysiologic phenomenon. The underlying dysfunction may involve deafferentation within the peripheral nervous system (e.g. neuropathy), deafferentation within the central nervous system (e.g. post-thalamic stroke) or an imbalance between the two (eg.phantom limb pain) (Cvijanovic *et al.,* 2011)

PERIPHERAL MECHANISMS:

Following a peripheral nerve injury (e.g. crush, stretch, or axotomy) sensitization occurs which is characterized by spontaneous activity of the neuron, a lowered threshold for activation and increased response to a given stimulus. Should the injured nerve be a nociceptor then increased nervous discharge will equate to increased pain. Following nerve injury C-fiber nociceptors can

develop new adrenergic receptors and sensitivity, which may help to explain the mechanism of sympathetically maintained pain. In addition to sensitization following damaged peripheral nerves, the formation of ectopic neuronal pacemakers can occur at various sites along the length of the nerve. Increased densities of abnormal or dysfunctional sodium channels are thought to be the cause of this ectopic activity (Schaible HG et al., 2007). The sodium channels in damaged nerves differ pharmacologically and demonstrate different depolarization characteristics.These ectopic pacemakers can occur in the proximal stump (eg.neuroma), in the cell bodies of the dorsal root ganglion, and in focal areas of demylenation along the axon. Neurogenic inflammation is a useful model for understanding pain and hyperalgesia. Neurogenic inflammation and the cascade of events following neural injury have been described. Inflammatory neuropeptides (substance P) and prostaglandins (PGE2) may be released from primary afferent nociceptors and sympathetic postganglionic neurons, respectively, activating nearby receptors and triggering a process of spreading activation. These mechanisms may explain the clinical response of some neuropathic pain patients to topical nonsteroidal anti-inflammatory drugs, lidocaine, and capsaicin. (Stephen M. Macres, Steven H. Richeimer and Paul J. Duran 2000)

CENTRAL MECHANISMS:

Following a peripheral nerve injury, anatomical and Neuro-chemical changes can occur within the central nervous system (CNS) that can persist long after the injury has healed. This "CNS plasticity" may play an important role in the evolution of chronic, neuropathic pain. As is the case in the periphery, sensitization of neurons can occur within the dorsal horn following peripheral tissue damage and this is characterized by an increased spontaneous activity of the dorsal horn neurons, a decreased threshold and an increased responsivity to afferent input, and

cell death in the spinal dorsal horn. In the non-injured state, A beta fibers (large myelinated afferents) penetrate the dorsal horn, travel ventrally, and terminate in lamina III and deeper. C fibers (small unmyelinated afferents) penetrate directly and generally terminate no deeper than lamina II. However, after peripheral nerve injury, there is a prominent sprouting of large afferents dorsally from lamina III into laminae I and II. After peripheral nerve injury, these large afferents gain access to spinal regions involved in transmitting high intensity, noxious signals, instead of merely encoding low threshold information. Significant alterations have been shown in the dorsal horn ipsilateral to the injury. The mechanisms are likely related to the barrage of afferent impulses or the factors transported from the lesion site. Therefore, any peripheral stimulation would activate a greater number of dorsal horn cells because of an increased overlap of their receptive fields. Evidence suggests that excessive nociceptive input to the dorsal horn can have excitotoxic consequences resulting in the death of inhibitory interneurons. This inhibition may contribute to spinal hyper-excitability. (Macres, et al., 2000)The allodynia and hyperalgesia associated with neuropathic pain may be best explained by:

1) The development of spontaneous activity of afferent input

2) The sprouting of large primary efferents (eg. A-beta fibers from lamina 3 into lamina 1 and

3) Sprouting of sympathetic efferents into neuromas and dorsal root and ganglion cells,

4) Elimination of intrinsic modulatory systems and

5) Up regulation of receptors in the dorsal horn, which mediate excitatory process.

Pathogenesis of inflammation

In vivo and *in vitro* experimental studies have identified biochemical pathways likely to be important in the development of inflammatory complications and have led to possible approaches to treatment.

The most studied mechanisms are

- TNF- α pathway
- NO pathway
- COX pathway
- Interleukin pathway

TNF- α:-

TNF- α known roles have extended from within the immune system to include a neuro-inflammatory domain in the nervous system Animal models of neuropathic pain based on various types of nerve injuries (peripheral versus spinal nerve, ligation versus chronic constrictive injury) have persistently implicated a pivotal role for TNF-α at both peripheral and central levels of sensitization (lawerence leung *et al.,* 2010) TNF-α plays a role in the peripheral mediation of neuropathic pain. Clinically, HIV therapy and chemotherapy produce peripheral neuropathy with massive release of TNF-α in serum (Tonini*et al.,* 2002)and TNF-α used as a clinical anti-cancer treatment leads to peripheral neuropathy (Drory*et al.,*1998). Traditional CCI of sciatic nerve in rats results in raised levels of TNF immunoreactivity in dorsal root ganglia (DRG) of both injured and uninjured ipsilateral adjacent afferents (Geis*et al*., 2003), as well as of contralateral uninjured counterparts (Jancalek*et al.,*2010), There is also a corresponding up-regulation of TNFR1 and TNFR2 in both nerve and DRG (Schafers *et al.,*2003),with a temporal pattern of increased TNF mRNA expression, First in sciatic nerve, and then in DRG (Franchi *et al*., 2008). When nucleus pulposus extract of the coccxygeal intervertebral disc is applied to lumbar DRG of rats ,neuropathic pain is induced but is abolished by co-application of TNRF1, implying a direct role of TNF as a local mediator(Cuellar *et al*., 2004). Exogenous TNF-α injected into DRG of CCI roots is transported both anterograde to the site of injury andretrograde

into the dorsal horn (Anterograde *et al*., 2002), precipitating allodynia in both the ligated and adjacent uninjured nerves (Lee*et al*., 2003). TNF-α is known to lead to apoptosis via TNFR1 (Thorburn *et al.*, 2003, Micheau *et al*., 2003) and the caspase signaling pathway (Micheau*etal.*, 2003). Caspase inhibitors can attenuate peripheral neuropathy experimentally induced byHIV therapy or chemotherapy in rats (Joseph *et al*., 2004). In addition to enhancing TTX-R Na^+ channels in nociceptive DRG neurons (Sekiguchi *et al*., 2009), TNF-α can also increase membrane K^+ ion conductance in a non-voltage-gated fashion (Joseph *et al*., 2004) leading to

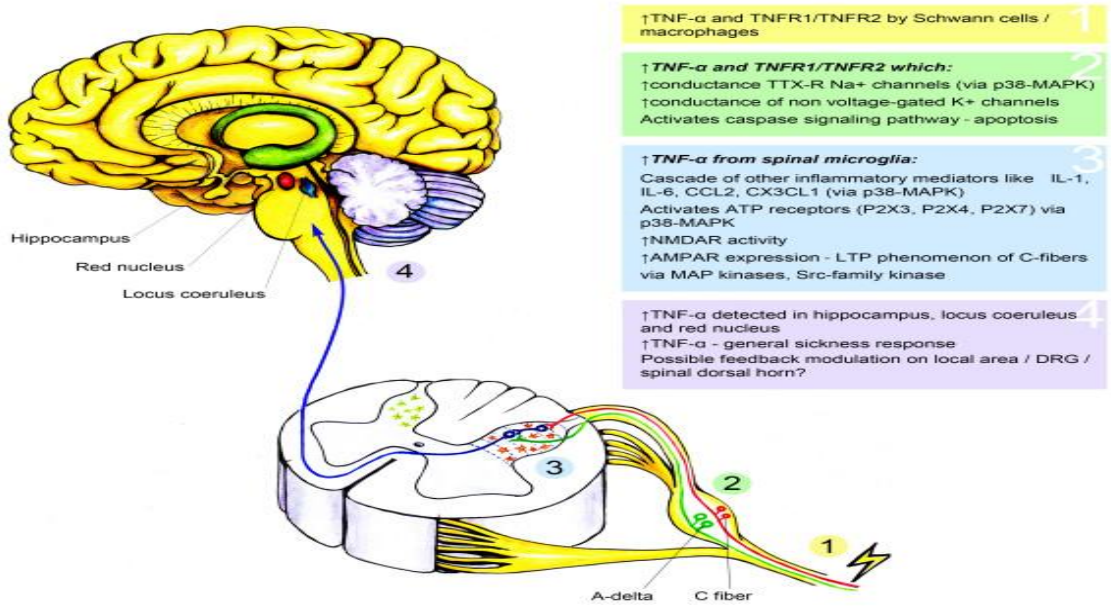


Figure 3: Mechanism of TNF-α pathway

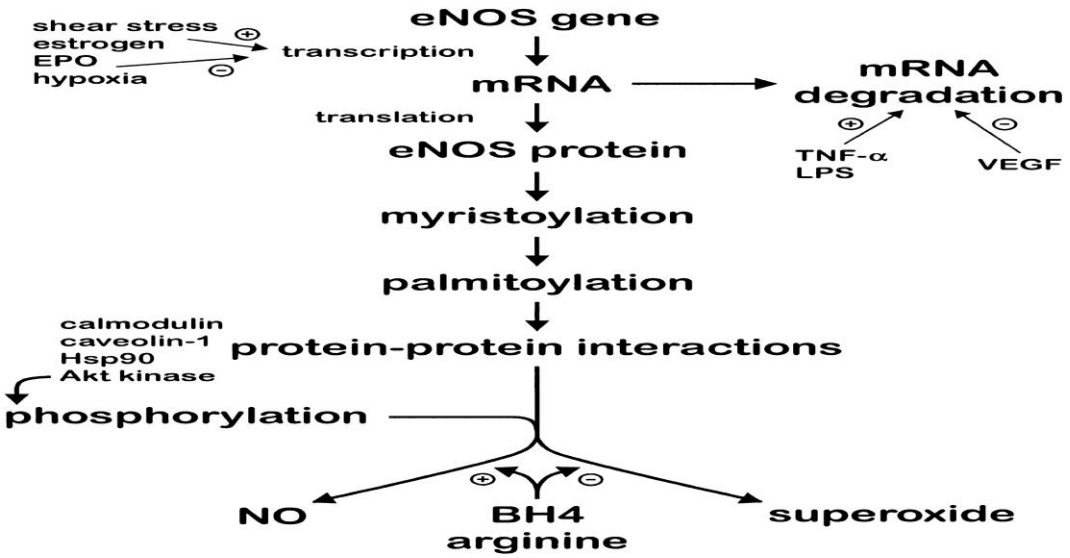
overall neuronal hyper-excitability and hence leading to neuropathic pain.TNF-α has received much attention as a culprit in neuropathic pain. Endoneurial administration induces hyperalgesia (Wagner and Myers., 1996). Preemptive treatment with the TNF-sequestering drug etanercept decreases hyperalgesia but has no effect once hyperalgesia is established (Sommer *et al*., 2001).

TNFα can initiate activity in nociceptors (Sorkin *et al*., 1997). Inhibition of TNF-α blocks phosphorylation of the MAP kinase p38 in DRG and hyperalgesia but again only when given preemptively (Schafers *et al*., 2003). Another cytokine that has received attention is IL-6. A role in the CCI model has been suggested (Okamoto *et al*., 2001)

Nitric Oxide:-

Nitric oxide (NO) is a free radical that produces a variety of biological actions under physiological and pathological conditions (Tannock IF *et al*.,2004)and is synthesized by three isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Reid AH et al., 2008)Nitric oxide (NO) is a short-living free radical that is produced from L-arginine by catalytic reaction of inducible NO synthases (iNOSs) within mammalian immune, cardiovascular, and neural systems, where it functions as a signalling or cytotoxic molecule and it is centrally involved in inflammation (Nathan *et al*., 1994). Among the three isomer NOSs reported to date, endothelial NOS (eNOS, NOS I) and neuronal NOS (nNOS, NOS III) are constitutive NOSs, whereas inducible NOS iNOS, NOS II can be induced by immunostimulatory cytokines and microbial products in various cell types, including endothelium, smooth muscle cells, hepatocytes, monocytes, mast cells, and macrophages (Nathan *et al*., 1994).Type-I nNOS (neuronal NOS) and Type-III eNOS (endothelial NOS) are constitutively expressed as latent enzymes and require a higher concentration of Ca2+ for the enzyme activity. In contrast, Type-II iNOS (inducible NOS) is Ca2+ independent because s high affinity for Ca2+/Calm (Calmodulin) renders the enzyme active even at basal levels of intracellular Ca2+ .The catalysis of this reaction require a number of essential cofactors such as mononucleotide, FAD (Flavin Adenine Dinucleotide), and NADPH (Nicotinamide Adenine Dinucleotide, Reduced). The NO thus generated exerts a number of functions in the cardiovascular system. Their interactions with

eNOS are stimulated by intracellular Ca2+ and lead to eNOS activation. Efficient supply with substrate during all this is ensured by localization of the arginine transporter CAT1 (Cationic Amino Acid Transporter-1) in caveolae and its direct interaction with eNOS.eNOS can interact with various proteins in its less active and more active states. Neuronal NOS (nNOS) is localized in a discrete population of neurones(Bredt *et al.,* 1991) and is dynamically regulated after peripheral inflammation (Herdegen *et al.,* 1994; Wu et al., 1998) Inducible NOS (iNOS) is mainly absent in neural tissues under normal conditions, but up-regulated in inflammation (Mungrue *et al.,* 2003), for example in astrocytes in the spinal cord dorsal horn (Maihofner *et al.,* 2000). Endothelial NOS (eNOS) is present in thebrain vasculature (Stanarius *et al.,* 1997) and also in astrocytes (Wiencken and Casagrande, 1999). Thus, all three NOS isoforms can act as sources of NO in the CNS after inflammation. The non-selective NOS inhibitor N-nitro--arginine methyl ester (L-NAME) reducesthermal hyperalgesia in inflammatory pain models (Semos and Headley, 1994; Osborne and Coderre,1999), indicating a role of NO in inflammatory pain. Furthermore, several reports indicated beneficial effects of selective blockers for nNOS and eNOS in reducing inflammatory and neuropathic pain,



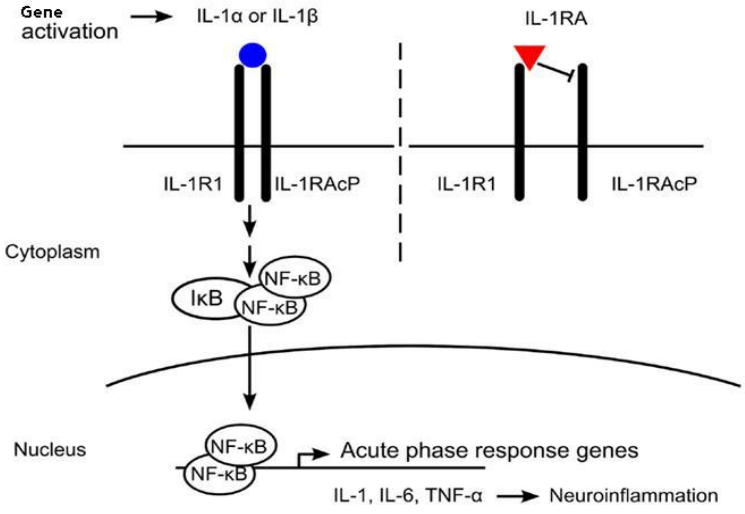
COX pathway:-

The arachidonic acid pathway is responsible for the generation of a wide variety of bio active metabolites. These metabolites, otherwise known as eicosanoids, have been shown to be involved in many different pathology, including inflammation. Arachidonic acid can be metabolized into the biologically active eicosanoids via the action of three separate groups of enzymes: cyclooxygenases (COX) also known as prostaglandin (PG) H synthase, lipoxygenases (LOX), and epoxygenases (cytochrome P450). The COX enzymes catalyse the first step in the synthesis of prostanoids from arachidonic acid (Wang et al., 2010). COX was shown to exist as two distinct isoforms in the early 1990s. These included the constitutively expressed COX-1 and the inducible form of COX-2, associated with inflammation (Warner et al., 2004). A third COX isoform has also been identified, known as COX-3 (Chandrasekharan et al., 2002). However, subsequent studies have shown that it has no COX activity and is therefore unlikely to have prostaglandin producing activity in human tissue (Snipes et al., 2005).

Interleukin:-

The interplay between the immune and nervous systems is thought to be critical for the development and maintenance of neuropathic pain, and the proinflammatory cytokines, including IL-1β, appear to be contributing to the pain state (Scholz and Woolf *et al.,* 2007, and Uceyler and Sommer *et al*., 2008). In various animal models of neuropathic pain, IL-1βexpression is increased in the injured sciatic nerve, DRG, and spinal cord (Rotshenker et al.,1992;Hashizume *et al*., 2000, Lee *et al*., 2004; Perrin *et al*., 2005; Ruohonen *et al*., 2005;Uceyler *et al*., 2007; Kawasaki *et al.,* 2008a).Immediately after peripheral nerve injury, Schwann cells are activated and macrophages are recruited to the injury site and both secrete IL-1β (reviewed in Scholz and Woolf*et al*., 2007).In a rat transected sciatic nerve model, upregulation of IL-1β has been

detected as long as 35 days post-surgery (Ruohonen*et al.*,2005). Recently, new mechanisms of neuropathic pain have been revealed involving a complex pathway with MMP9, MMP2 and IL-1β. Kawasaki et al., 2008ashowed that in the PSNL model cleavage of IL-1β by MMP subtypes contributed to different phases of neuropathic pain behavior. Therefore, IL-1β is likely part of a complex signaling cascade involving MMPs in the CCI model. Nerve injury increases expression and secretion of proinflammatory cytokines, including IL-1β, IL-6, and interferon-γ, all of which are required for the development of pain hypersensitivity (Murphy *et al*., 1995; Costigan *et al*., 2009). Interleukin-6 is an inflammatory cytokine with wide-ranging biological effects. It has been widely demonstrated that neuro inflammation plays a critical role in the development of pathological pain. Recently, various pathological pain models have shown elevated expression levels of interleukin-6 and its receptor in the spinal cord and dorsal root ganglia. This sensitization triggers the production of mediators, alteration of ion channels, and sprouting of nerveendings.



Many evidences have indicated that IL-6 plays a critical role in neuropathic pain caused by PSNL induced peripheral neuropathic pain. IL-1 is particularly known to modulate pain sensitivity. IL-1 has also been implicated in the development of neuropathic pain. Elevated levels

of IL-1 were detected following peripheral nerve injury (DeLeo *et al*., 1997; Rotshenker *et al*., 1992; Shamashet *et al*., 2002). peripheral hyperalgesic effects of IL-1 could be mediated directly by exciting nociceptive fibers, or indirectly by stimulating the production of pronociceptive compounds, such as nitric oxide (NO), prostaglandins, and nerve growth factor (NGF), or the release of calcitonin gene related peptide (CGRP) (Marchand *et al*., 2005)

CHRONIC PAIN AND MEMORY

To understand the connection between chronic pain and memory problems or other forms of cognitive problems, this fact is that the experience of pain occurs because of our brain and other parts of our nervous system. Whatever the initial cause of pain, once the pain becomes chronic; it becomes a nervous system problem. Different areas of the nervous system, such as in the brain and spinal cord, go through changes and these changes are responsible for the development of chronic pain. One area of the brain that appears to go through such changes is the hippocampus (Cardoso-Cruz, Lima, & Galhardo *et al.,* 2013; Mutso, *et al.,* 2013). The hippocampus is involved in the development of central sensitization (Lamtremoliere & Woolf, 2009), depression (Campbell & MacQueen, 2004), and memory (Squire, 1992). Pain can change the hippocampus. In turn, these changes to the hippocampus can create a cascading effect on the experience of pain, leading to central sensitization, depression, and memory problems. Much is yet to be learned from the study of chronic pain and the nervous system. The hippocampus may not be the only thing that is responsible for the development of memory problems in people with chronic pain.

Animal models of neuropathic pain:-

Animal models are pivotal for understanding the mechanism of neuropathic pain and development of effective therapy for its optimal management. A battery of neuropathic pain

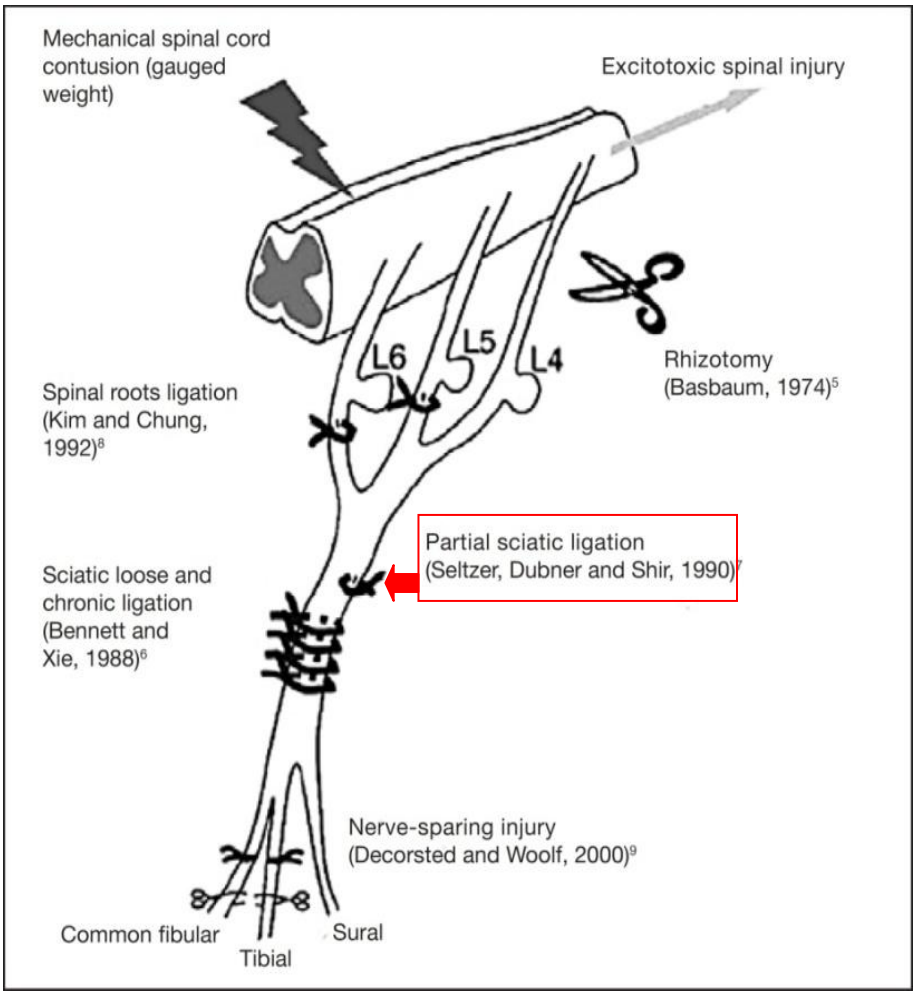
models has been developed to simulate the clinical pain conditions with diverse etiology. As each animal model has been created with specific methodology and results tend to vary largely with the slight changes related to methodology, therefore, it is essential that data from different models should be reported and interpreted in the context of the specific pain model

(Jaggi *et al.,* 2011)

Partial sciatic nerve ligation

Partial nerve injury is the main cause of causalgic form pain disorders in humans. Here present here a novel animal model of this condition. In rats we unilaterally ligated about half of the sciatic nerve high in the thigh. In this model, the left hind leg of a rat is shaved and dissection is made to expose the sciatic nerve at the upper-thigh level. The dorsal one-third to half of the sciatic nerve is tightly ligated with an 8–0 silk suture just distal to the point at which posterior biceps semitendinosus nerve branches off. The behavioral signs of spontaneous pain in the form of paw guarding and licking on the injury side have been reported. The behavioral alterations like cold allodynia, chemical hyper-reactivity, and mechanical hyperalgesia have been noted to occur within 1 week after the surgery and most of the changes persist for 6 weeks PSNL model represents sympathetically independent pain (SIP) in the first week post-operation. (Dowdall *et al.,* 2005.Within a few hours after the operation, and for several months thereafter, the rats developed guarding behavior of the ipsilateral hind paw and licked it often, suggesting the possibility of spontaneous pain,(seltzer *et al.,* 1990) The plantar surface of the foot was evenly hyperesthetic to non-noxious and noxious stimuli.. The partial nerve injury models are relevant for understanding neuropathic pain injury, as the partial nerve injury is the main cause of causalgic form pain disorders in humans. (Seltzer et al., 1995) Because of the immediate onset and long-lasting continuation of touch-evoked allodynia and hyperalgesia (parallel to the pain in

humans with causalgia), it has been suggested that PSNL model is a good animal model for causalgic form pain syndromes (Malmberg *et al.*, 1998)



Behavior models for neuropathic pain and memory:

The hot-plate test is useful for evaluating acute pain. The hot plate test involves two types of responses: paw licking and jumping. Both responses integrate at supraspinal structures with the C and A-delta type I and II sensitive fibers participating in this model (Lopes *et al.*, 2009). The nociceptive withdrawal threshold was assessed by using the Randall-Selitto electronic algesimeter. The Randall-Selitto test appears to be a useful method for studying neuropathic pain conditions affecting both the fore- and hind limbs, and thus responses corresponding to above

and below the segmental level of the lesion. Central sensitization, providing a mechanistic explanation for how low threshold A or C fibers can begin to transmit pain, has been generally accepted (Chu *et al*., 2008).This commercially available instrument (electronic von Frey) is similar to that successfully used to quantify neuropathic allodynia (Bach *et al*., 1994) and inflammatory hyper nociception in rats (Li *et al*., 1998). Performance on this task is assessed by measuring the animal's latency to enter the goal box at the end of the beam. The assessment of motor function with the beam-balance and beam-walking tasks have been the primary dependent variable in the study of the neuro pharmacologic mechanisms of peripheral injury (Hayes *et al*.,1988; Lyeth *et al.*,1988). The rotarod task, first developed by Dunham and Miya (1957), is a frequently used screening test for evaluating neurologic effects of various drugs. As originally used, the procedure required the rat to maintain its equilibrium on a rod that rotated at a constant speed. Jones and Roberts (1968) increased the sensitivity of the task by gradually\ increasing the speed that the rod rotated while the animal was walking on the rod. Several reports have investigated the effects of CNS depressants on rotarod performance and have found the device to be a useful tool for evaluating motor function (Kuribar *et al.*,1977).However, the rota rod task has not been explored as a potential instrument for identifying motor deficits following by peripheral injury Morris water-maze performance involves several components, including concept formation (learning the general rules of the task), attention, working memory, and reference memory, which are not readily distinguishable in a simple form of this paradigm (Morris,1989; Whishaw, 1989; Bannerman et al., 1995). Thus, we used several variants of the Morris water-maze in an effort to deter- mine whether the rat lines differed in their associative abilities, conceptual abilities, working and reference memory. Search strategies, and native attention distractibility. The novel object recognition task has been widely used for studies on

interferences in the recognition memory (Ennaceur and Delacour, 1988). The task is based on the spontaneous behavior of the animals to explore more a novel object than a familiar one. Thus, this task has been widely used to assess normal physiological events as well as genetic and pharmacological interventions in the recognition memory process (Chuhan and Taukulis, 2006; de Bruin and Pouzet, 2006; Palchykova et al., 2006).

Plant profile

Desmodiumgangeticum (Shalparni) is a 2-4 feet high erect under shrub with woody stem, branches covered with soft hairs. It is found in the forest and waste lands of India, from the plains and Western Ghats, and up to 1500 m in the north up to Sikkim and also in south India. Shalaparniplant leaves are unifoliate, ovate, oblong, obtuse, and pubescent beneath and up to 15 * 8 cm long. Its inflorescence is many flowered, terminal or auxiliary, slender and 15-30 cm long. The flowers are small and of pink-violet colour. Pods are thin, pointed, 1.5 to 2 cm long clothed with minute hooked hairs. Flowering and fruiting occur in the month of August and November. (Subha Rastogi et al., 2011)

Scientific Classification

The botanical name of is *Desmodiumgangeticum* (L.) DC. It belongs to plant family Fabaceae. Fabaceae, is also known as Leguminosae or pea family and consists of about 20,000 species of plants, including trees, shrubs, vines, and herbs distributed all across the world. The fruits are present inside a legume or seed pod. (Yadav *et al.*, 2013)

Constituents of *Desmodium gangeticum*

The roots contain several alkaloids, flavonoids, pterocarpanoids— gangetin (gives antiinflammatory activity), gangetinin, and desmodin. The aerial parts contain indole-3-alkylamines and their derivatives. (Shri Vijaya Kirubha *et al.*, 2011)

Ayurvedic Properties and Action of *Desmodiumgangeticum*:-

The root of the plant is astringent, bitter and sweet in taste (Rasa), sweet after digestion (Vipaka), and is hot in effect (Virya).It is an Ushna Virya herb. UshnaVirya or hot potency herb, subdues Vata (Wind) and Kapha (Mucus) and increases Pitta (Bile). It has a property of digestion, vomiting and purging, and gives a feeling of lightness.(Pantulu *et al.,* 1948)

Important Medicinal Properties

Desmodiumgangeticum is rich in medicinal properties. The understanding of these properties will help us to better utilize this herb.The medicinal plant Shalparni or Sarivan (*Desmodiumgangeticum*) is extensively used in Ayurveda. It is one of the ten roots of famous anti-inflammatory formulation of Ayurveda, Dashmula. Dashmula has been used since time immemorial to treat swelling inside body and intake of Dashmula balances Vata and Kapha, digests the ama and gives relief in pain. For medicinal use purpose whole plant is used. (Sagar *et al.*,2010)

Below is given medicinal properties along with the meaning, (Rastogi *et al.,* 2011)

Antioxidant: neutralize the oxidant effect of free radicals and other substances.

Anti–inflammatory: reducing inflammation by acting on body mechanisms.

Anti–nociceptive: inhibits nociception, the sensation of pain.

Anti–rheumatic: alleviating or preventing rheumatism.

Anti–osteoarthritic: Against Osteoarthritis.

Astringent: causing the contraction of body tissues.

Antipyretic / antifebrile / febrifuge: Effective against fever.

Anthelmintic: anti-parasitic, expel parasitic worms (helminths) and other internal parasites from the body.

Diuretic: Promoting excretion of urine/agent that increases the amount of urine excreted.

Immunostimulant: stimulate the immune system by inducing activation or increasing activity of any of its components. (Suman singh et al., 2016)

Chapter 3

Objective & Plan of study

3. PLAN OF STUDY

SPECIFIC OBJECTIVE

- To prepare Desmodium *gangeticum* aqueous extract and to quantify the chemical constituents through HPTLC fingerprinting study.

- To assess the effect of *Desmodium gangeticum* in LPS induced inflammation in RAW 264.7 (Murine macrophage) cell lines.

- To find the activity of the *Desmodium gangeticum* extract on mechanically induced Pain and cognitive impairment in partial sciatic nerve ligation rat model.

- To find the mechanism of action of *Desmodium gangeticum* extract by measuring inflammatory markers in sciatic nerve and brain through conventional PCR , ELISA and histopathological studies also been done to support the research findings.

PHAES: I

Extraction of the Herb

- Aqueous extract preparation through water decoction
- Qualitative analysis by chemical test
- Quantification of chemical constituents by HPTLC

PHAES: II

In-vitro Studies

- Dose Optimization of DG extract as well as LPS by MTT assay and the effect of DG Extract towards LPS induced toxicity by co-administering LPS and DG extract.

PHAES: III

PSNL Surgery

- Partial Sciatic Nerve Ligation was done in rats to induce neuropathic pain through mechanically in rats, Treatment was continued up to 28^{th} day and general observation was done on 7^{th}, 14th, 21st & 28^{th} day using behavioral test models such as thermal, mechanical, chemical induced pain model, cognitive functions in rats was recorded by performing Morris water maze and novel object recognition tests.

PHAES: IV

Measurement of pro-inflammatory mediators & Histopathology studies

- At 28^{th} day sciatic nerve was isolated for histopathological studies and measuring pro-inflammatory mediators responsible for neuropathic pain like TNF-α, IL-1β, IL-6, e-NOS and COX were measured through PCR and ELISA studies.

Chapter 4

Materials & Methods

4. MATERIALS AND METHODS

4.1 Chemicals and Instruments Used

The chemicals used for the present study were tabulated.

Table 1: List of chemicals used

Chemicals	*Manufacturer*
Ketamine	Med India
Xylenol	Kruz Pharma
Carboxy Methyl Cellulose	OTTO
Toluene	RANKEM
HPTLC Plates	Merck
Millipore water	Direct-Q
Lipopolysaccharide	Sigma Aldrich
3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide	Sigma Aldrich
Dimethyl sulphoxide	Fisher Scientific
PCR KIT	HI-MEDIA
ELISA LIT	HI-MEDIA
Ethylenediamine tetra acetic acid	HI-MEDIA
Gabapentin	Sun Pharma

Table 2: List of instruments used

Instrument	*Manufacturer*
Laminar Airflow	AIRSYS
Inverted Trinocular Phase contrast Microscope	Motic
CO_2 Incubator	SANYO
Refrigerated centrifuge	Eppendrof
Deep freezer	Cryo scientific systems
Autoclave Sterilizer	Everflow scientific
UV-Visible spectrophotometer	Schimadzu
Vortex	TARSONS
PCR	Thermo Scientific
HPTLC	CAMAG
ELISA READER	Thermo Scientific

4.2 Animals

Adult female Sprague Dawley rats (200-250g) were obtained from the animal house of PSG IMS&R, Coimbatore. The rats were grouped and housed in polypropylene cages (38 X 23 X 10cm) with not more than 6 animals per cage. They were maintained at an ambient temperature of 25$\pm$1°C. Animals had free access to food and water. The animals were acclimatized to laboratory condition for one week prior to experiments. The experimental protocols were approved by Institutional Animal Ethics Committee and conducted according to the guidelines of the Committee for the Purpose of Supervision and Control of Experiments on Animals (CPCSEA), Government of India.

4.3 *Desmodium gangeticum* Water Decoction Extract

In this *Desmodium gangeticum* ***aqueous*** extract prepared by water decoction method as per Ayurvedic principle.The shade-dried root and aerial parts were powdered and passed through 10-mesh sieve. The coarsely powdered materials (1000 g) were soaked in distilled water in the ratio of 1:16 (w/v). The extracts were filtered, pooled and first concentrated on rota vapour and then dried in freeze dry system/Freezone® 4.5 with high vacuum, at −40 ◦C (yield 14.1% root and 16.7% (w/w) aerial parts of dried extract). The chemical constituents of the root and aerial decoction were identified by qualitative analysis and confirmed by thin layer chromatography (Trease and Evans, 1983). This indicates the presence of alkaloids and flavonoids. For the pharmacological tests, the dried extract of root and aerial parts of *Desmodium gangeticum* was suspended in double distilled water containing 1% (w/v) carboxymethyl cellulose (CMC) and were used throughout our experimental studies.

4.3.1 Qualitative Chemical Tests

Test for alkaloids:-

Mayer's test

Extracts or fractions were treated with Mayer's reagent (Potassium-mercuric Iodide), appearance of cream colored ppt. confirmed alkaloids.

Wagner's test

Extracts or fractions were treated with Wagner's reagent (Iodine in potassium iodide solution), when appearance of reddish brown ppt. confirmed alkaloids.

Dragendorff's test

Extracts or fractions were treated with Dragendorff's reagent (Solution of Potassium Bismuth Iodide), when appearance of reddish brown ppt. confirmed alkaloids.

Test for Carbohydrates:-

Molisch's test

Extracts or fractions were treated with α-naphthol followed by addition of Conc. Sulphuric acid, when appearance of purple colour confirmed the presence of carbohydrates.

Benedict's test

Extracts or fractions were treated with Benedict Reagent
(173gm sod. Citrate + 100gm sod. Carbonate + 17.3gm copper sulphate) followed by boiling for 2 min then allowed to cool, when appearance of red, yellow or green ppt. depending on amount of sugar confirmed the presence of carbohydrates.

Tests for Flavonoids and Coumarins:-

Mineral acid test

Extracts or fractions were treated with Conc. Sulphuric acid, when appearance of yellow orange colour confirmed the presence of Flavonoids.

Sodium Hydroxide test

Extracts or fractions were treated with 10% sod. Hydroxide solution, when appearance of yellow orange colour confirmed the presence of Flavonoids.

Test for steroids:-

Libermann-Burchard Sterol reaction

To the extracts or fractions glacial acetic acid was added followed by addition of conc. Sulphuric acid, when a colour change from rose, through red, violet and blue to green confirmed the presence of steroids.

Salkowski reaction

To the extracts or fractions chloroform was added followed by addition of conc. Sulphuric acid, when appearance of red colour in chloroform layer confirmed the presence of steroids.

Test for triterpenoids

Hirchorn test

To the extracts or fractions Trichloroacetic acid was added followed by warming, when appearance of yellow to red colour confirmed the presence of triterpenoids.

Salkowski reaction

To the extract or fractions chloroform was added followed by addition of conc. Sulphuric acid, when appearance of red colour in chloroform layer confirmed the presence of triterpenoids.

Test for tannins

Ferric chloride test

To the extracts or fractions freshly prepared Ferric chloride solution was added, when appearance of blue-black or brownish colour confirmed the presence of tannins.

4.3.2 HPTLC finger printing

The samples were spotted in the form of bands of width 6 mm with a CAMAG microlitre syringe on precoated silica gel aluminium plate 60 F–254, (10 cm × 10 cm with 250 µm) thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai. By using a CAMAG Linomat IV(Switzerland). The plates were prewashed by methanol and activated at 60°C for 5 min prior to chromatography. A constant application rate of 0.1 µl/sec was employed and space between two bands was 5 mm. The slit dimension was kept at 5 mm × 0.45 mm and 10 mm/sec scanning speed was employed. The monochromatic band width was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of (Ethyl acetate: glacial acetic acid: Formic acid water (100:11:11:26) and 15 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 10 cm × 10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) saturated with the mobile phase and the chromatographic plate development was carried out for two times with the same mobile phase. The optimized chamber saturation time for mobile phase was 1hr at room temperature (25 ± 2 °C) at relative humidity of 60 ± 5%. The length of chromatogram run was 8 cm. Subsequent to the development, HPTLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed on CAMAG TLC scanner III in the reflectance-absorbance mode at 366 nm and operated by CATS software (V 3.15, CAMAG). The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was via peak areas with linear regression. (Himani Agrawal *et al*., 2004).

Chromatographic condition

Stationary phase	:	Silica Gel GF254
Mobile phase	:	Ethyl acetate: glacial acetic acid: Formic acid water(100:11:11:26)
Chamber saturation time	:	1 hr
Instrument	:	HPTLC (CAMAG-version 1.3.4, USA)
Applicator	:	Linomat V
Scanner	:	CAMAG TLC Scanner
Developing mode	:	Ascending mode (multiple development)
Detection reagent	:	Dragendorff's reagent
Scanning wavelength	:	254, 366 nm
Experimental condition	:	25±2˚C Temp/RH: 55-65%

***4.4In vitro* studies**

4.4.1 Mammalian cell culture

RAW 264.7 (Murine macrophage cell line) was obtained from NCCS Pune. It was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% serum(FBS), amphotericin (3 µg/ml), gentamycin (400 µg/ml), streptomycin(250 µg/ml) and penicillin (250 units/ml) at 37°C in a carbon dioxide incubator at 5% CO_2.

4.4.2 Preparation of media and other reagents required for cell culture

4.4.2.1 Minimum Essential Medium (MEM) was prepared as follows

The powdered media was dissolved in 900 ml of sterile glass - distilled or Millipore water in an autoclaved glass conical flask under sterile conditions. The antibiotics were added in the concentration as mentioned above and stirred well. 3.7 g of Sodium bicarbonate was added into the flask and stirred until it gets dissolved completely. 10% FBS was added and mixed well. The liquid was slowly poured into the upper portion of a Media sterilisation unit (Corning) and filtered through a 0.2µ filter under negative pressure. The medium was immediately stored at $4^{o}C$.

4.4.2.2 Saline: Trypsin: Versene (STV*)*

10X Saline A: 8g NaCl, 0.4g KCl, 1.0g D-Glucose and 0.35g $NaHCO_3$ (Tissue Culture Grade) were dissolved in 100 ml water and stored at 4^oC. *Versene:* 1g EDTA (Tissue Culture grade) was added into 90 ml distilled water. Then 5N NaOH was added drop wise until it gets dissolved. The solution was filter sterilized and stored at 4^oC. 100 ml of STV was prepared by adding 25 mg of trypsin in a mixture of 10 ml of 10X Saline A and 2.5 ml of Versene Double distilled water was added to make up to 100 ml, sterile filtered, liquated, and frozen at $–20^oC$ to $–70^oC$.

4.4.2.3Maintenance and storage of cell lines

RAW 264.7 cells show a steady growth rate with a doubling time of 36 to 48 hrs. The cells reached confluence in 6 to 7 days and these cells were passaged to get the cells for the experiments and also to store in liquid nitrogen. Passaging was done as follows:The culture medium was removed from the 25 ml culture flask by decanting into a clean container inside the laminar airflow chamber, and cells were rinsed with medium without serum, to remove traces of serum, which may inhibit action of trypsin. 2 ml of STV solution was added to the flask containing cells and incubated at 37^oC for a few minutes. As soon as cells started dislocating from the surface, flask was rinsed with 5ml of serum-containing medium to arrest the trypsinisation. The suspension of cells was collected in a sterile 15 ml centrifuge tube and the cells were pelleted at 1500rpm for 3 minutes. The cell pellet was resuspended in fresh medium with serum and a part of the cells were seeded back into the flask. The remaining cells were used for experiment or pelleted as earlier and resuspended in cryopreservative medium (10% DMSO in serum) in a cryovial (Nunc) and frozen at -70^oC for a day then transferred to liquid nitrogen.

4.4.4 Drugs and LPS Preparation

Extracts were subjected to solubility test with different organic solvents, finally get dissolved in dimethyl sulphoxide (DMSO), 25 mg/100 µl stock was prepared. The desired doses such as of 0.01 to 0.5µg/ml were prepared from the stock using culture medium. Lipopolysaccharide (LPS) in the dose of 1mg/ml was prepared with distilled water.

4.4.5 MTT assay

RAW 264.7 cell lines were maintained in Minimum Essential Medium (MEM) supplemented with 10% serum (FBS), amphotericin (3µg /ml), gentamycin (400µg/ml), streptomycin (250 µg /ml) and penicillin (250 units/ml) in a carbon dioxide incubator at 5% CO_2. 5000-10000 cells/well were added in 96 well plates from well grown culture medium, the viability was tested using trypan blue dye with help of haemocytometer and 95% of viability was confirmed. After 24 hrs, the LPS (1- 400µg/ml) were incubated with Cultured RAW cell lines separately for 24 hrs. After incubation, the LPS containing medium was removed and the medium was changed again for all groups and 10 µl of MTT (5 mg/ml stock solution) was added and the plates were incubated for an additional 4 hrs. The medium was discarded and the formazan blue, which was formed in the cells, was dissolved with 150 µl of DMSO. The optical density was measured at 595 nm. The percentage toxicity was calculated by using following formula

% Toxicity = (LPS treated cells/LPS untreated cells) x 100

***In vivo* studies**

Table 3: Treatment schedule

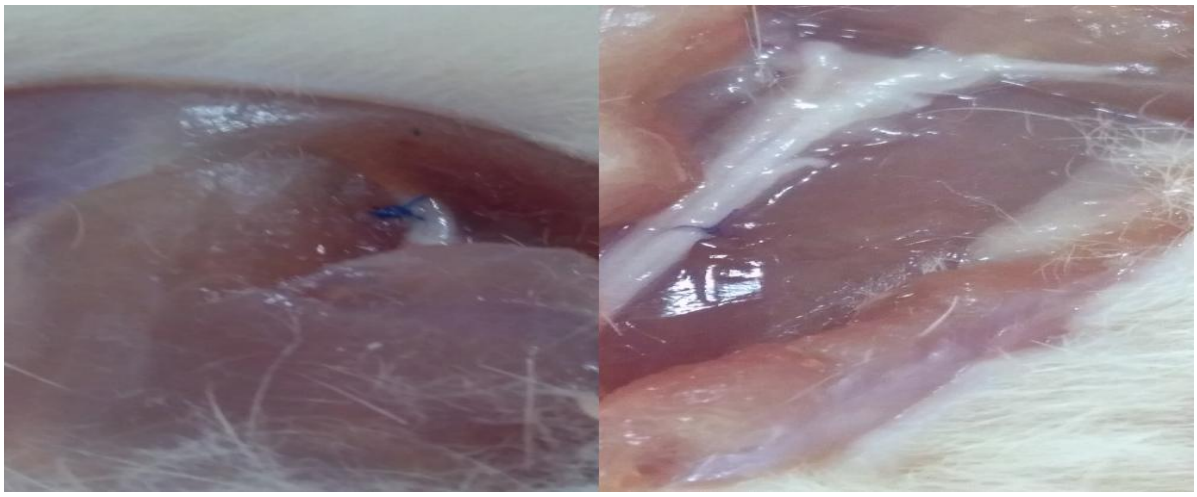
GROUP	TREATMENT	NUMBER OF ANIMALS
Group-I	Sham control	6
Group-II	Negative control	6
Group-III	Standard control (gabapentin 30mg/kg)	6
Group-IV	PSNL+ *Desmodium gangeticum* (25mg/kg, p.o.)	6
Group-V	PSNL+ *Desmodium gangeticum* (50mg/kg, p.o.)	6

4.5 PSNL Induction for rats:-

The animals were divided into five groups.1st group is designated as grouped as sham operated (rats were underwent surgical procedure without nerve ligation) and other rats from remaining group underwent partial sciatic nerve ligation. Treatment was started after 24hrs of the surgery with gabapentin (30mg/kg), *Desmodium gangeticum* extract *(*25 & 50mg/kg*)*, up to 28 days. The drug effect was observed on 7th and 28th day using thermal, mechanical and chemical induced pain assessment and also other cognitive function tests.

4.5.1 Partial Sciatic Nerve Ligation Model:

Peripheral neuropathic pain after partial sciatic nerve ligation was induced in SD rats (female) using a similar procedure to that described by Seltzer *et al.* for rats. Rats were anesthetized with xylazine (50mg/kg) and ketamine (120 mg/kg) (intraperitoneal) Unilateral mono neuropathy was induced by a rigid side ligation on one side exposed by making a skin incision, and cutting through the connective tissue between the gluteus superficialis and biceps femoris muscles of about one- third to one-half of the left sciatic nerve (ipsilateral) diameter performed with 8–0 silk suture. To just occlude but not arrest epineural blood flow. The wound is closed with sutures in the muscle and staples in the skin.. The ligatures were loosely tied until a short flick of the ipsilateral hind limbwas observed. The animal is then allowed to recover from surgery for 24 hrs before pain hypersensitivity testing begins. However the recovery time may vary from 2nd day to 2 weeks time as the intensity of pain lasts for about 3 weeks or one month time according to various studies. The best time for the pain hypersensitivity testing is found to be testing the subject in periodic intervals starting from 7th day, up to the 28th day postoperative as during this time the hypersensitivity reaches the peak with a previous testing of half an hour prior to the surgery and 2 days before surgery. The size of the nerve to be freed from adhering tissues is 7 mm. So, that the length of the nerve tied with ligatures and affected will be 4-5 mm long. The dosing of the rats with the test drug will be done after 24 hours of recovery from surgery which may continue till maximum 28 days after which the ligated sciatic nerve and other required tissues will be isolated from the sacrificed rat and will be subjected for histopathology analysis and other estimation studies (seltzer et al., 1990).



4.5.2 Hot plate method (THERMAL HYPERALGESIA*):*

In this test, the animals were individually placed on a hot plate (Eddy's hot plate) with the temperature adjusted to 55_1 _C. The latency to the first sign of paw licking or jump response to avoid the heat was taken as an index of the pain threshold; the cut-off time was 10 s in order to avoid damage to the paw (Tiwari *et al*., 2011)

4.5.3 Randell-sellito method (MECHANICAL HYPERALGESIA):

This paw pressure test was conducted according to the method described by Woode et al 72 and Zakaria et al 73. The rats were placed on the Ugo Basile dynamic plantar aesthesiometer for 20 minutes prior to the experiment to enable them to adapt to their surroundings constant force of 20 g/s was applied to the injected paw with cutoff time at 30 seconds, and the response time for nociceptive behavior (withdrawal of the injected paw) was recorded .

4.5.4 Von-frey filaments (MECHANICAL ALLODYNIA):

To measure mechanical thresholds, the electronic Von Frey method was used. Rats were first weighed on a scale before being placed in separate plexiglass boxes on top of a raised wire mesh. They were left to acclimate for approximately 30 minutes prior to testing. Stimulation of the lateral area of the hind paws was done using the 90 g arm and the size 8 filament. The maximum threshold readout that accompanied a paw flick was recorded. Both paws of each animal were stimulated three times, with 2-minute rest in-between stimulations.

4.5.5 COGNITIVE BEHAVIOURAL MODELS:

4.5.5.1 MORRIS WATER MAZE:

Morris water maze employed in the present study was a model to evaluate spatial learning and memory (118). It consisted of large circular pool (150 cm in diameter, 45 cm in height, filled to a depth of 30 cm with water maintained at 28±1^{o}C). During the MWM test, a platform 15 cm in diameter was located 1.5 cm below the water in one of four sections of the pool, approximately 50 cm from the sidewalls. The pool was divided into four quadrants of equal area. A camera was mounted to the ceiling above the pool and the paths taken by the rats were recorded.

Acquisition phase

The MWM test was initiated on the 3rd day after gabapentin administration had started. The animals received four trials per day. The rats were trained to find the hidden platform. The position of platform was kept unaltered throughout the training session. Each animal was subjected to four consecutive training trials on each day. The rat was gently placed in the water between quadrants, facing the wall of pool and allowed 60 sec to locate submerged platform. Then, it was allowed to stay on the platform for 20 sec. If it failed to find the platform within 60 sec, it was guided gently onto platform and allowed to remain there for 20 sec. Day 6 escape latency time (ELT) to locate the hidden platform in water maze was noted as an index of acquisition or learning. Animals were subjected to training trials for six consecutive days. Between one trail and the next, water was stirred to erase olfactory traces of previous swim patterns.

Retention phase

On ninth day, the platform was removed and each rat was allowed to explore the pool for 90 sec. Mean time spent in the target quadrants was noted. The mean time spent by the animal in target quadrant searching for the hidden platform was noted as an index of retrieval or memory. The time to reach the target quadrant and the number of crossings the animal made while searching the target quadrant was also noted as index of memory.

4.5.5.2 Novel-object recognition test

The novel-object recognition test procedure Testing was carried out in a circular arena with a wooden base and metal sides (both painted black), with dimensions 75 cm (d) ×38 cm (h). The arena was illuminated by four 60 W bulbs which provided constant light intensity of 100 lx. The objects used were with a base diameter of 4.5 cm and 23.5 cm height and a plastic structure constructed from green and pink toy blocks with dimensions base area 5 cm2 and height 16 cm. The objects had no apparent natural significance to the rats, and were secured to the base of the arena with white tack such that they were difficult to displace. Animals were habituated to the arena in the absence of objects for 20 min on the day before the test day.

The test day comprised of three stages:

(i) Habituation, (ii) exposure one and (iii) exposure Two

Rats were introduced to the arena for a 3 min habituation period and then returned to their home cage for 7 min. During exposure 1, two identical objects were placed in opposite quadrants of the arena, 16 cm from the perimeter. The rat was allowed to freely explore the arena and objects for a period of 3 min, after which the animal was removed from the arena and returned to its home cage for an interval of 5 min. prior to exposure 2, one of the object was replaced with a novel object.

The animal was again allowed to freely explore the arena and objects for a period of 3 min and then returned to its home cage. The arena was cleaned with a mild detergent between rats to remove odours and olfactory cues, and fecal pellets were removed between exposures.

Exploration of an object was defined as sniffing the object, rearing against the object or having the head directed towards the object within a 2 cm annulus of the object. Exploratory and general behaviors were manually rated of each of the three test stages, the proportion of time spent exploring the object was assessed by calculating a discrimination ratio as follows:

Assessments;

An inspection is operationally defined as approaching, sniffing and looking at object from a distance of 2cm

Habituation

Time taken to initially enter the innerzone time spent in centrezone

Training

Time taken to initially enter and inspect the objects time taken inspecting each object

Time spent in center zone

Testing

Time taken to initially enter and inspect the objects Time taken inspecting each object

(50% of time inspecting the familiar suggests memory deficit)

Entries into centre

Note; sitting on object not considered an inspection

Percent time inspecting; TF/TN+TF*100

[TF=spent exploring familiar object; TN=time spentexploring the novel object]

4.6 Conventional PCR:

Total RNA was isolated by Trizol method as per manufacturer protocol. The pellet was washed once with 75% ethanol centrifuged again for 5 min, air-dried and dissolved in 50 µlDEPC treated water. Total RNA was stored at -80ºC. 1µg of RNA was using Enhanced Avian C-DNA Kit (Sigma Aldrich,USA). After initial denaturation for 10 min at 95°C, thirty five amplification cycles were performed for TNF-α (30s of 95 °C denaturation,1 min of 60 °C annealing and extension), IL1β (15s of 95 °C denaturation, 1 min of 60 °C annealing and extension.

4.7 ELISA:

Rat TNF-a and IL-1b, IL-6 levels were estimated using ELISA kits in sciatic nerve according to the manufacturer's instructions (R&D Systems, USA). Protein estimation was done by Bradford method and 100µg of proteins (lysed in lysis buffer) diluted in reagent diluents up to 100µl were taken for the assay as suggested by the protocol guideline.

4.8 Histopathological measurements*:*

The histopathological examination of the myelin sheath level in the sciatic nerve will be carried out to assess the extent of nerve degeneration.

4.9 Statistical analysis

Data were given in the form of arithmetical mean values and standard error mean. The collected data were subjected to one way ANOVA (Analysis of Variance) followed by *posthoc* analysis Bonferrni multiple comparison test for MTT Assay. One way ANOVA followed by Bonferroni post test was used for comparing behavior models p value of less than 0.05 were considered as significant. The analysis was carried out by using Graph pad prism software of version 5.03.

Chapter 5

Results

5. RESULTS

5.1 Phytochemical analysis of *Desmodium gangeticum*

Table:5

S.No	Phytochemicals	Presence /absence
1.	Alkaloids	+++
2.	Flavanoids	+++
3.	PHYTOSTEROL	+
4.	Tannins	++
5.	Saponins	++

Phytochemical screening:

+++: Present in high concentration, ++: Present in moderate concentration and +: Present in low concentration

5.2 HPTLC Finger printing

Finger printing of *Desmodium gangeticum* extract,was done using HPTLC and spots were visualized at 254 and 366 nm. The solvent system used for development of chromatogram was ethyl acetate: glacial acetic acid:formic acid:water (100:1.1:1.1:26, (v/v/v/v). The brown colour spots obtained in chromatogram (Fig. 1) shows the presence of flavonoids in extract of Desmodium gangeticum. The presence of flavonoids was confirmed by derivitization with Dragendorff's reagent. Quercetin was used as reference standard. A defined peak at Rf=0.34 for the standard flavonoid quercetin was obtained. The 3D display of the densitogram and chromatogram indicates the presence of quercetin like phytochemical in *Desmodium gangeticum* extract.The limit of detection was found to be 20ng/spot on siliga gel $60F_{254}$ and the linearity

range were validated in the range of 1-4 ug . The quantity of quercetin present in the *Desmodium gangeticum.* was found to be 0.608mg/gm of extract (Fig 2)

5.3 In vitro models

Effect of extracts treatment on cell viability in LPS treated RAW 264.7 cells

Treatment of RAW 264.7 cell line with aqueous extract of *Desmodium gangeticum* showed no significant decrease in cell viability in comparison to solvent treated cells. In comparison to solvent treated cells (100% viable cell), LPS 72 µg/ml treated cells showed significant ($p<0.01$) decrease in cell viability. Treatment of aqueous extract of *Desmodium gangeticum* with LPS at concentration 0.001 – 0.5 µg/ml showed significant protection of RAW 264.7 cell line ($p<0.001$) in comparison to LPS treated cells. (Fig 3)

5.4. In vivo models

5.4.1 Hot plate method

In comparison to sham operated group, Partial Sciatic Nerve Ligation (PSNL) operated groups showed significant reduction in escape latency on 7^{th} ,14^{th} 21^{th} ($p<0.001$) and 28^{th} day ($p<0.05$)

Gabapentin (30mg/kg) treated rats showed significant recovery of hyperalgesic response on 7^{th}, 14^{th}, 21^{th}, 28^{th} day as observed by increased basal latency period in comparison to PSNL group ($p<0.001$). *Desmodium gangeticum* (25 & 50mg/kg) treated rats showed significant ($p<0.001$) increased in basal latency period on 7^{th}, 14^{th}, 21^{st}, 28^{th} days as compared to PSNL group. *Desmodium gangeticum* (25 mg/kg) treated rats has shown a decreased basal latency period as compared to Gabapentin (30mg/kg) treated rats. *Desmodium gangeticum* (50mg/kg) treated rats has shown no significant difference as compared to Gabapentin (30mg/kg) treated rats indicates that *Desmodium gangeticum* (50mg/kg) is effective as gabapentin. (Fig 4)

5.4.2 Randell-sellito method

Partial Sciatic Nerve Ligation (PSNL) operated group showed significant reduction in mechanical hyperalgesia in comparison to sham operated group, on ,14 th ($p<0.001$), 21th ($p<0.001$) and 28th day ($p<0.05$) Gabapentin (30mg/kg) treated rats showed significant recovery of hyperalgesic response on 7th, 14 th,21th, 28 th day as observed by increased mechanical threshold in comparison to PSNL group ($p<0.001$). *Desmodium gangeticum* (25 mg/kg) treated rats showed significant increase in mechanical threshold on 14 th($p<0.05$), 21th ($p<0.001$), 28 th ($p<0.001$), days as compared to PSNL operated group. Similar results were observed with Desmodium *gangeticum* (50 mg/kg). *Desmodium gangeticum* (50mg/kg) treated rats did not show significant difference with gabapentin (30mg/kg) treated rats indicates *Desmodium gangeticum* (50mg/kg) is equally efficacious as gabapentin (Fig 5)

5.4.3 Electronic von Frey

In comparison to sham operated group, (PSNL) groups showed significant reduction in mechanical allodynia on 14 th ($p<0.01$), 21th ($p<0.05$) and 28th day ($p<0.01$)

Gabapentin (30mg/kg) treated rats showed significant increase in mechanical allodynia on 7th, 14 th, 21th, 28 th day as observed by increase pain threshold level in comparison to PSNL rats ($p<0.001$).

5.4.4 ROTA ROD TEST:

PSNL rats showed significant neurological deficits in comparison to sham-operated rats ($p<0.001$) as observed decreased time spent in rota rod test. Gabapentin (30mg/kg) treated rats showed a improvement ($p<0.001$) by increase retention time in the rotarod test on 1st week. *Desmodium gangeticum* (25&50mg/kg*)* treated rats showed improved rotarod retention time ($p<0.001$) in the rotarod test. (Fig 7)

5.5 MORRIS WATER MAZE:

Effect on time spent in the target quadrant:

The rats significantly spent more time in the target quadrant in search of missing platform as compared to the time spent in other quadrants during retrieval trial conducted on day 7, indicating memory loss or retrieval difficulty. In PSNL group markedly reduced time spent time in target quadrant (Q4) in search of missing platform during retrieval trial reflects impairment of memory. PSNL group had a significant decrease ($p<0.001$) in time spent in target quadrant in comparison to control rats. Gabapentin (30mg/kg) and *Desmodium gangeticum* (25mg&50mg/kg) treated rats showed significant increase ($p<0.001$) in time spent in target quadrant in comparison with to PSNL rats. Treatment with *Desmodium gangeticum* (25 & 50mg/kg) exhibited dose dependent increase ($p<0.001$) in time spent in target quadrant in comparison to PSNL treated rats. *Desmodium gangeticum* (50 mg/kg) treated rats has shown a significant reduction in time spent in target quadrant as compared to gabapentin (30mg/kg) treated rats ($p<0.001$) (Fig 8.1)

Effect on time to reach the target quadrant:

The decrease in time to reach the target quadrant indicates the memory of the animal in searching the missing platform. PSNL resulted in significant increase ($p<0.001$) in time taken to reach the target quadrant in comparison to control rats. Gabapentin (30mg/kg), *Desmodium gangeticum* (25&50mg/kg) treatment showed significant decrease ($p<0.001$) in time taken to reach the target quadrant in comparison to PSNL treated rats. *Desmodium gangeticum* (25 &50mg/kg) treatment exhibited dose dependent ($p<0.001$) effect. (Fig 8.2)

Effect on number of crossings:

During retrieval trial, if the rat crosses the target quadrant many times in search of platform it indicates the retention of memory. PSNL surgery in rats resulted in significant decrease ($p<0.001$) in number of crossings in the target quadrant in comparison to control rats. Treatment with gabapentin (30mg/kg), *Desmodium gangeticum* (25mg/kg&50mg) showed significant increase ($p<0.001$) in number of crossings in the target quadrant in comparison to PSNL treated rats. gabapentin 30mg/kg, *Desmodium gangeticum* (25mg/kg&50mg/kg) administered rats exhibited dose dependent increase ($p<0.05$) in number of crossings of rats in the target quadrant in comparison to vehicle treated PSNL rats.(Fig 8.2)

5.6 EFFECT ON NOVEL OBJECT RECOGNITION TEST:

Time taken to initially enter and inspect objects

The control rats significantly reach the each objects as compared to PSNL group during trial conducted on day 4 indicates intact cognitive functions. PSNL rats take more time to reach the each objects during the retrieval trial reflects impairment of memory.

The PSNL rats spent significantly less time in exploring the novel object. This observation clearly indicates PSNL rats had cognitive deficits in the novel object recognition test. PSNL rats, resulted in significant decrease ($p<0.001$) in time to reach and inspect the objects compare to sham operated rats. Gabapentin (30mg/kg), *Desmodium gangeticum* (25 &50mg/kg) treatment showed significant increase ($p<0.001$) in time spent in target quadrant in comparison to PSNL treated rats. (Fig 9.1)

Time taken inspecting each objects (familiar object &novel object)

PSNL in rats resulted in increased time spent on familiar object comparison to other groups showing memory deficits and also in significant decrease ($p<0.001$) in time to reach the novel

object comparison to sham rats. Gabapentin (30mg/kg), *Desmodium gangeticum* (25mg&50mg/kg) treatment showed significant increase in ($p<0.001$) time spent in novel object comparison to PSNL treated rats. (Fig 9.1)

Percentage time spending:-

In comparison to sham operated group, PSNL operated group showed significant increase in percentage time spending in familiar object respectively ($P<0.001$). This indicates PSNL operated rats spent more time in familiar objects due to cognitive impairment. Treatment with gabapentin (30mg/kg), *Desmodium gangeticum* (25mg/kg&50mg) showed significant reduction in percentage time spending in familiar object as compared to PSNL operated groups ($p<0.001$) respectively.

5.5 Effect on pro-inflammatory mediators quantified by conventional PCR:-

5.5.1COX measurement

PSNL in rats resulted in significant increase ($p<0.001$) in pro-inflammatory mediator COX mRNA level when compared to control rats. Gabapentin (30 mg/kg) significantly ($p<0.001$) decreased COX mRNA level in comparison to PSNL rats. *Desmodium gangeticum* (25 & 50mg/kg) treated rats showed significant ($p<0.05$) decrease in COX mRNA levels (Fig 10.1)

5.5.2 e-NOS measurement

PSNL in rats resulted in significant decrease ($p<0.001$) in pro-inflammatory mediator. e-NOS level in comparison to control rats. Gabapentin (30 mg/kg) significantly ($p<0.001$) increase the pro-inflammatory mediator level in comparison to PSNL rats. *Desmodium gangeticum* (25 & 50mg/kg) showed significant ($p<0.05$) increase in e-NOS levels (Fig 10.2)

5.5.3 Effect on pro-inflammatory mediators quantified by ELISA:-

PSNL in rats exhibited a significant increase in TNF-α, levels at ($p<0.00$) in comparison to sham operated rats. PSNL exhibited significant increase in IL-1β and IL-6 ,the level ($p<0.05$) in comparison to sham operated rats. Gabapentin treated rats showed significant ($p<0.01$) reduction in the IL-1β and ($p<0.001$) IL-6 level in comparison to PSNL rats. *Desmodium gangeticum* (25mg/kg) treated rats showed significant ($p<0.01$) reduction in IL-1β level and ($p<0.001$) IL-6 level in comparison to PSNL rats. *Desmodium gangeticum* (50mg/kg) treated rats significantly ($p<0.05$) reduced the TNF-α, IL-1β and IL-6 level ($p<0.001$) in comparsion to PSNL rats. Comparison to gabapentin group IL-1β and IL-6 showed significant reduction ($p<0.05$) at *Desmodium gangeticum* (50mg/kg) .

5.6 Histopathalogical studies

The axonal degeneration was evident in the negative control group (fig.11.2) as compared to the sham operated group (fig.11.1). The morphological picture shows normal axons on the standard group (fig11.3) and treatment groups (fig11.4&11.5) when compared to negative control rats shows decreas areas of degenerated fibers. By the end of the study period after 28 days there was axonal regeneration in the gabapentin treated rats and *Desmodium gangeticum* (25& 50mg/kg) treated rats. The axonal regeneration was found to be better in *Desmodium gangeticum* (25&50mg/kg) treated rats in comparison to PSNL rats and also, PSNL surgery in rats showed more deposition of collagen fibers which was indicative of fibrosis. The axonal regeneration in gabapentin (30mg/kg) treated rats was less than the sham and PSNL operated rats.

HPTLC analysis

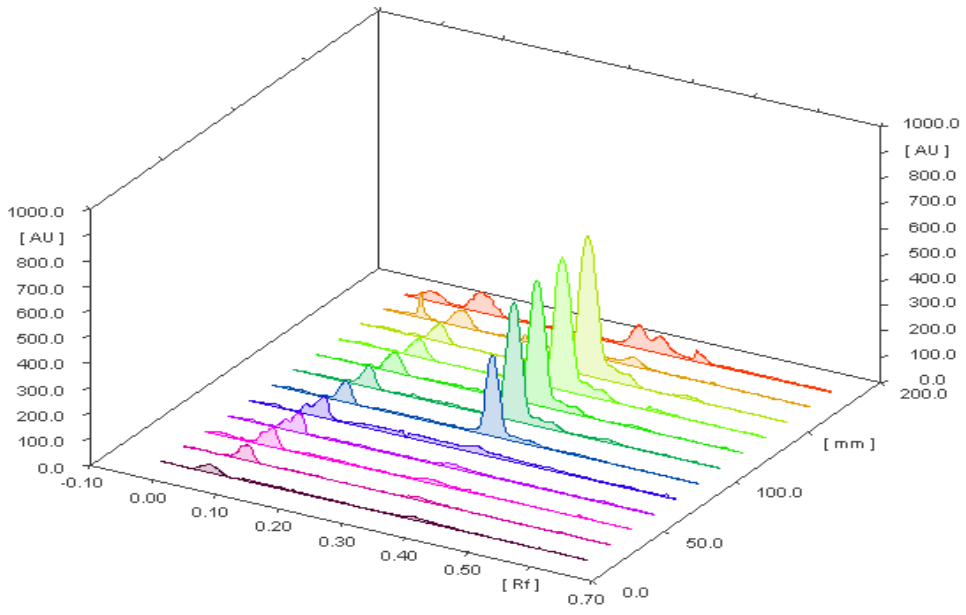


Figure 1: 3D value of quercetin and desmodium gangeticum

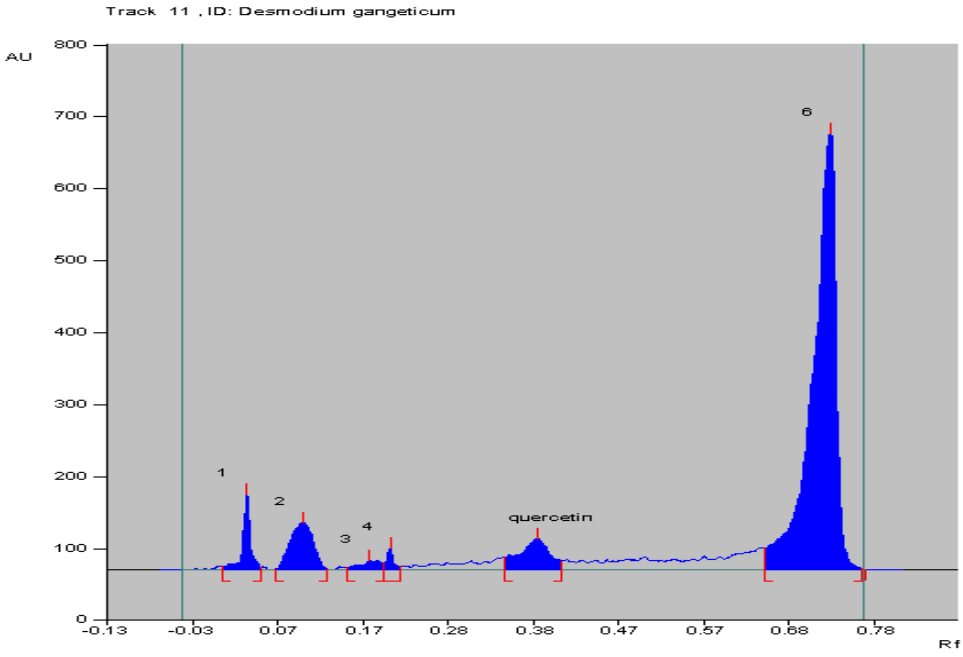


Figure 2: R_f value of quercetin and desmodium gangeticum

Effect of *Desmodium gangeticum* on LPS treated RAW 264.7 cell lines.

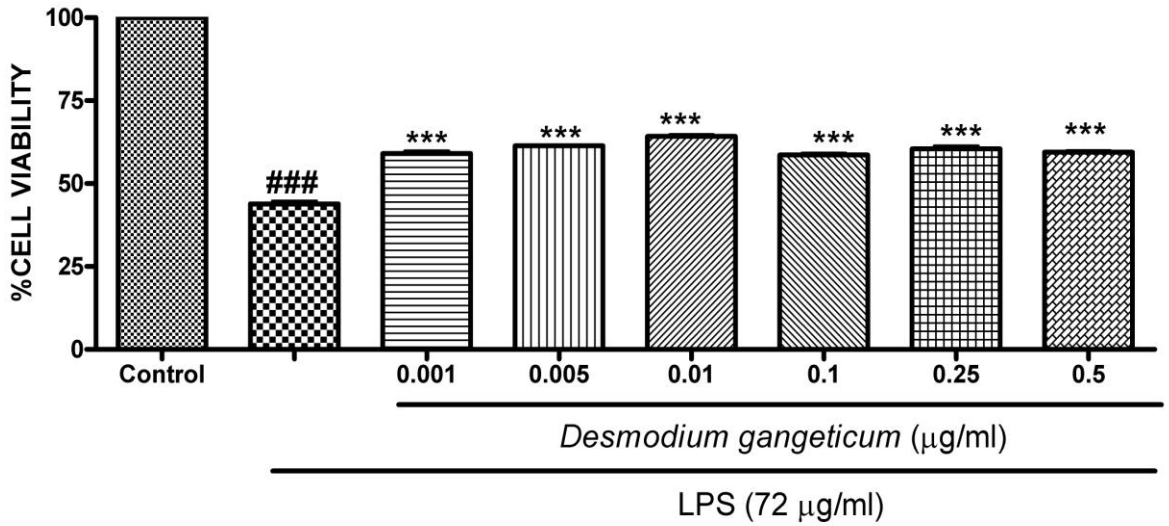


Figure 3. Effect of *Desmodium gangeticum* on cell viability in LPS treated RAW 264.7 cell lines.LPS= Lipopolysaccharide All the data were expressed as mean ± SD. One way ANOVA followed by post-hoc tukey comparison test. $^{###}$**,** denotes the statistical significance in comparison to control at p<0.001 respectively.*****,** denotes the statistical significance in comparison to LPS treated cells at p<0.001, respectively.

Effect of *Desmodium gangeticum* on PSNL induced neuropathic pain in rat

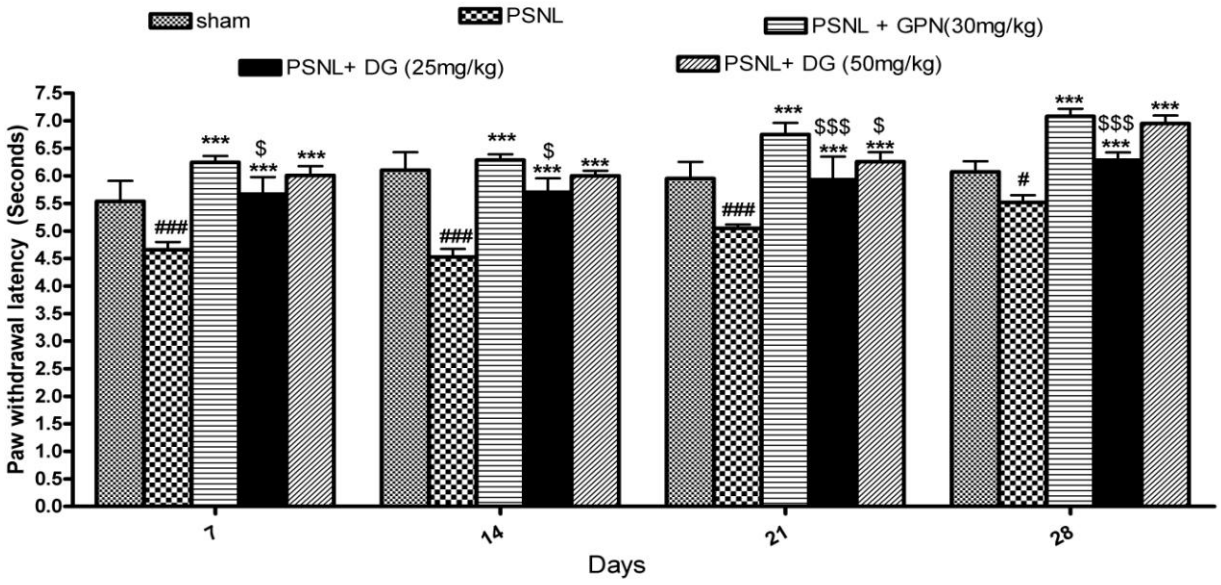


Figure 4. Effect of various treatments on hotplate test

All the data were expressed as mean ± SD. Two way ANOVA followed by post hoc bonferrni multiple comparison test. PSNL=Partial sciatic nerve ligation GPN=Gabapentin DG=*Desmodium gangeticum.* $^{\#}$, $^{\#\#}$, $^{\#\#\#}$,denotes statistical significance in comparison to sham treated group at p<0.05, p<0.01, p<0.001 respectively. ***,**,***** denotes statistical significance in comparison to PSNL treated group at p<0.05, p<0.01, p<0.001 respectively. $^{\$}$, $^{\$\$}$, $^{\$\$\$}$ denotes statistical significance in comparison to GPN treated group at p<0.05, p<0.01, p<0.001 respectively.

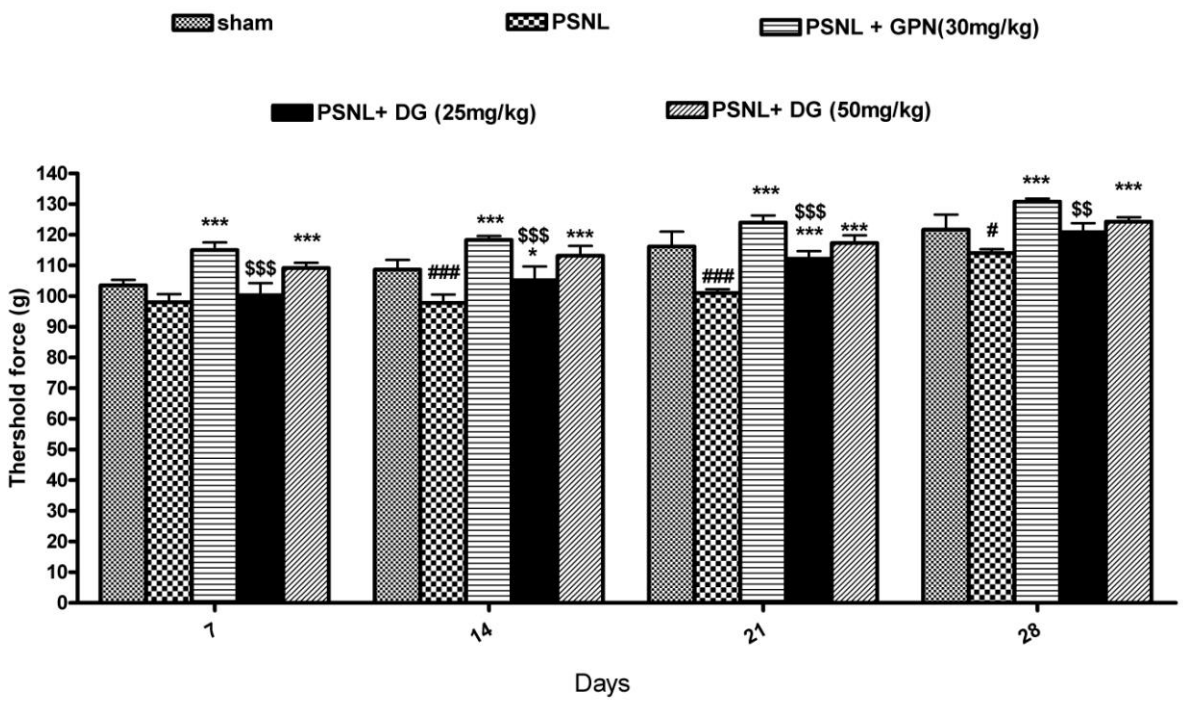


Figure 5 Effect of various treatments on randal selito test

All the data were expressed as mean ± SD. Two-way ANOVA followed by post hoc bonferrni multiple comparison test. PSNL=Partial sciatic nerve ligation. GPN=Gabapentin DG=*Desmodium gangeticum* $^{\#}$, $^{\#}$, $^{\#\#\#}$,denotes statistical significance in comparison to sham treated group at $p<0.05$, $p<0.01$, $p<0.001$ respectively. ***,**,***** denotes statistical significance in comparison to PSNL treated group at $p<0.05$, $p<0.01$, $p<0.001$ respectively. $^{\$}$, $^{\$\$}$, $^{\$\$\$}$ denotes statistical significance in comparison to GPN treated group at $p<0.05$, $p<0.01$, $p<0.001$ respectively.

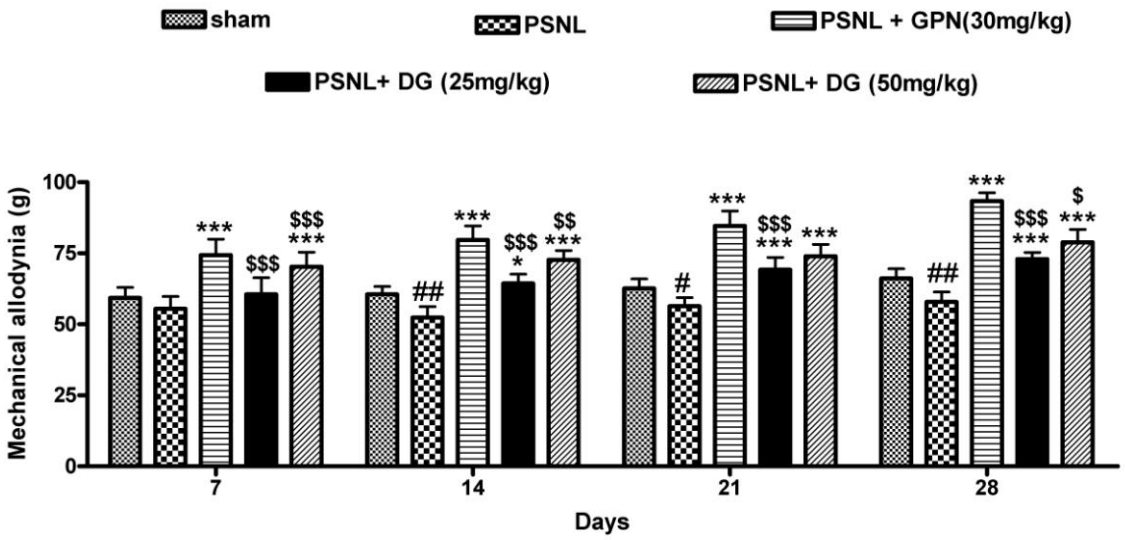


Figure 6 .Effect of various treatments on von frey test

All the data were expressed as mean ± SD. Two way ANOVA followed by post hoc bonferrni multiple comparison test. PSNL=Partial sciatic nerve ligation .GPN=Gabapentin DG=*Desmodium gangeticum* $^{\#}$, denotes statistical significance in comparison to sham treated group at $p<0.05$ respectively. ***,**,*** ,** denotes statistical significance in comparison to PSNL treated group at $p<0.05$, $p<0.01$, $p<0.001$ respectively. $^{\$}$, $^{\$\$}$, $^{\$\$\$}$ denotes statistical significance in comparison to GPN treated group at $p<0.05$, $p<0.01$, $p<0.001$ respectively

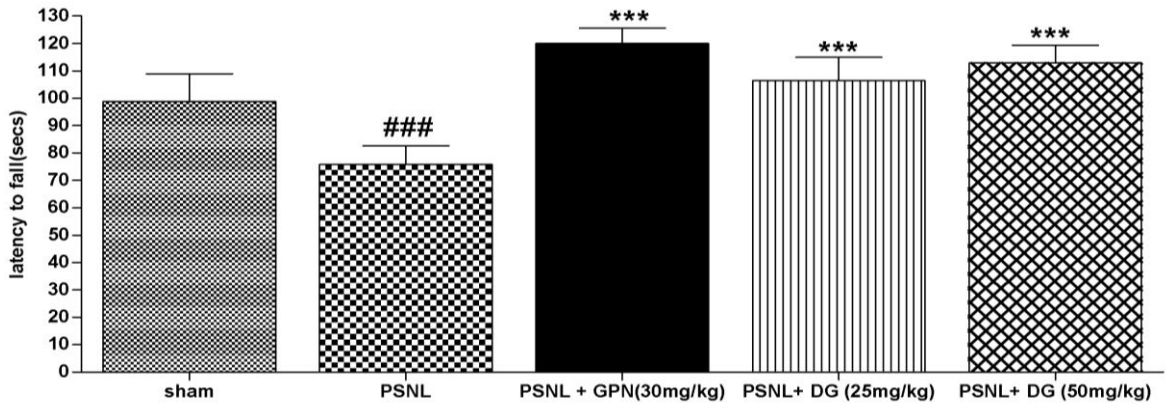
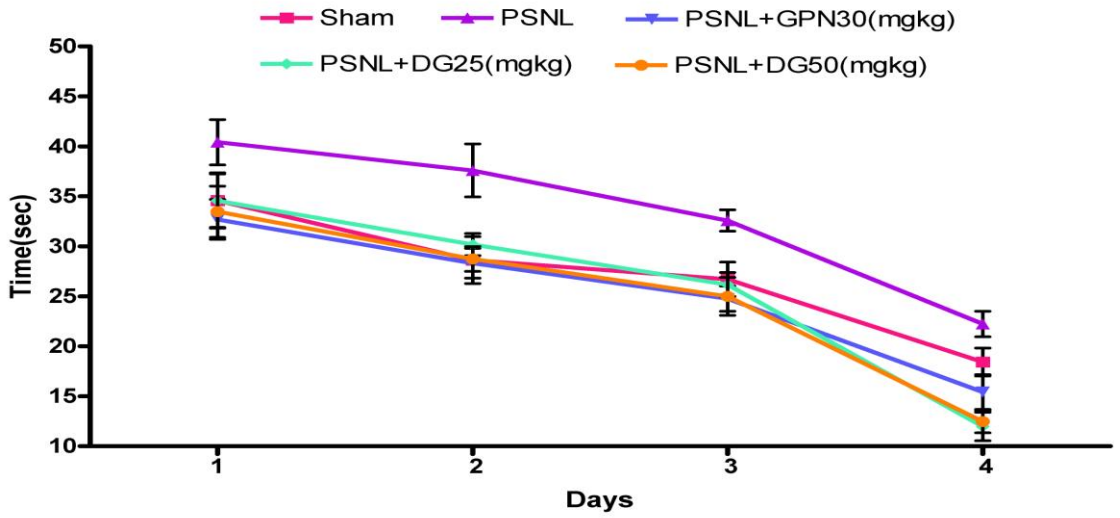


Figure 7. Effect of various treatments on rota rod test

All the data were expressed as mean ± SD. One way ANOVA followed by post -hoc tukey comparison test. PSNL=Partial sciatic nerve ligation GPN=Gabapentin DG=*Desmodium gangeticum* ###**,** denotes statistical significance in comparison to sham treated group at $p<0.001$ respectively. *****,** denotes statistical significance in comparison to PSNL treated group at $p<0.001$ respectively.

Training



Effect on time spent in the target quadrant:

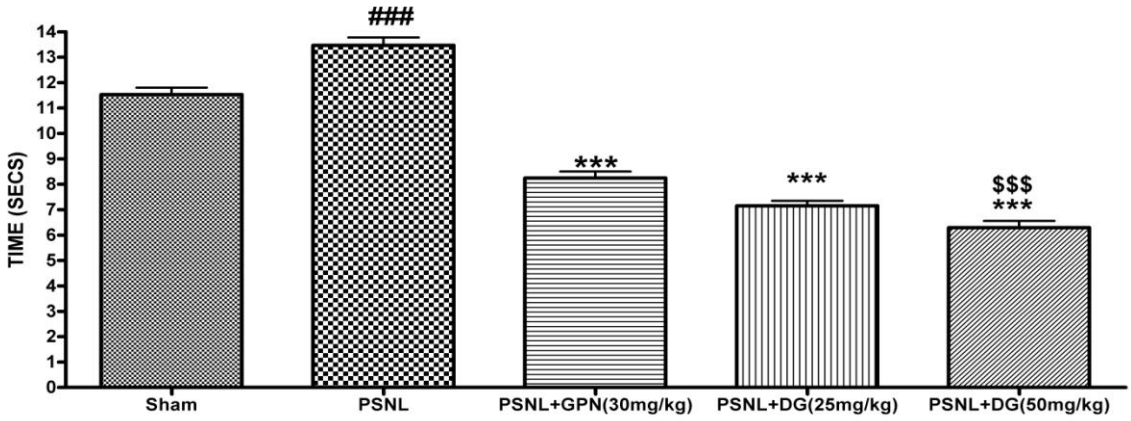
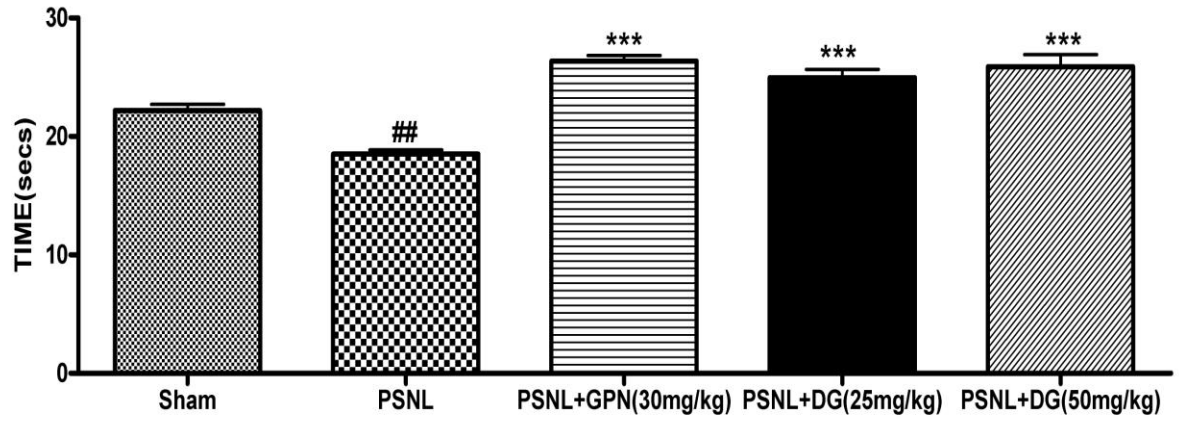


Figure 8.1: Effect of various treatments on morris water maze test

All the data were expressed as mean ± SD. One way ANOVA followed by post hoc tukey comparison test. PSNL=Partial sciatic nerve ligation GPN=Gabapentin DG=*Desmodium gangeticum* $^{###}$**,** denotes statistical significance in comparison to sham treated group at p<0.001 respectively. *****,** denotes statistical significance in comparison to PSNL treated group at p<0.001 respectively $^{\$\$\$}$**,** denotes statistical significance in comparison to GPN treated group at p<0.001 respectively

Time to reach target quadrant:-



No of crossing:-

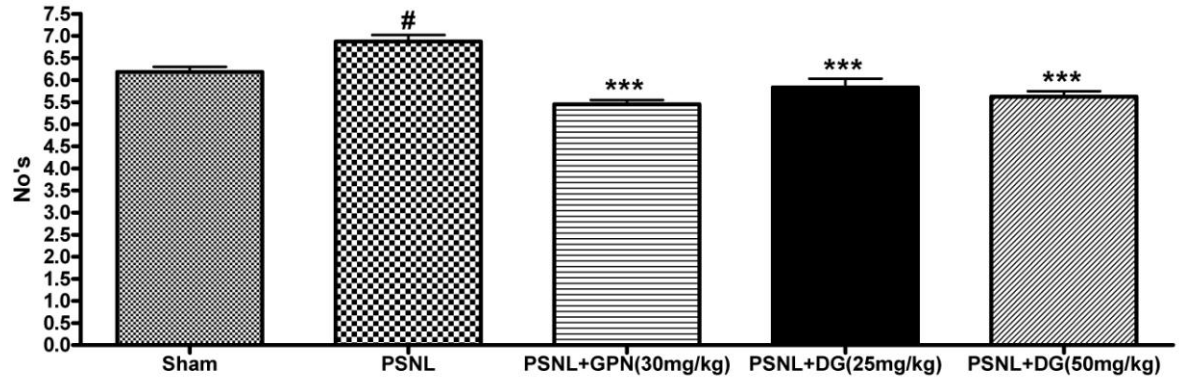
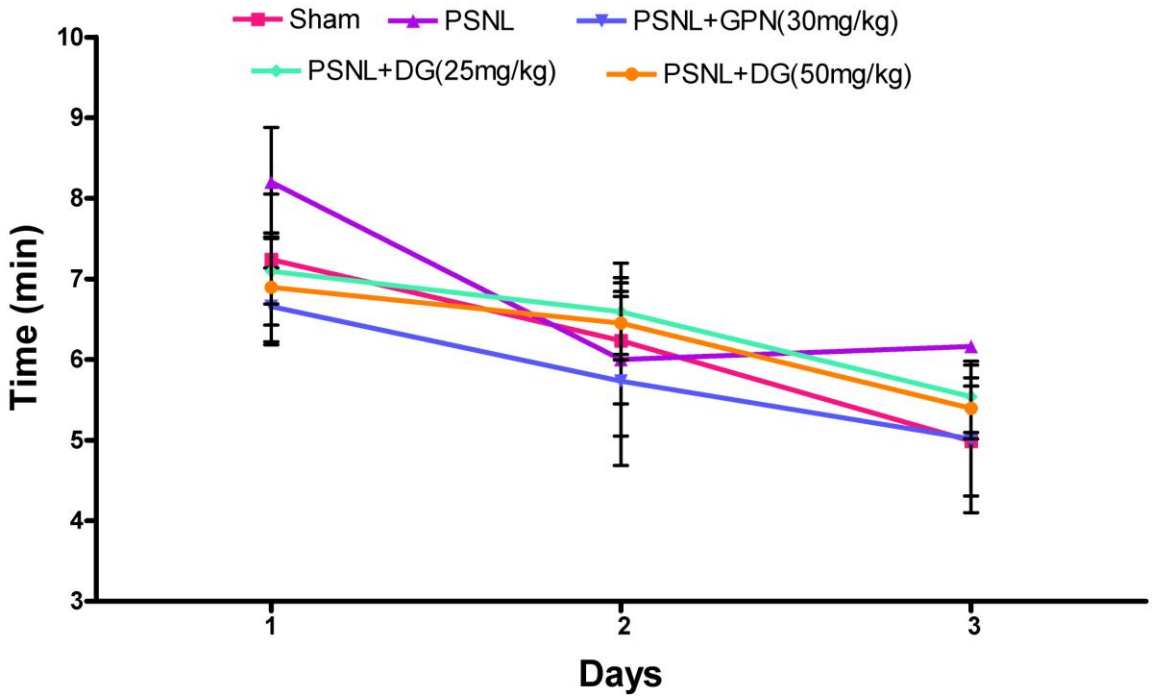


Figure 8.2: Effect of various treatments on morris water maze test

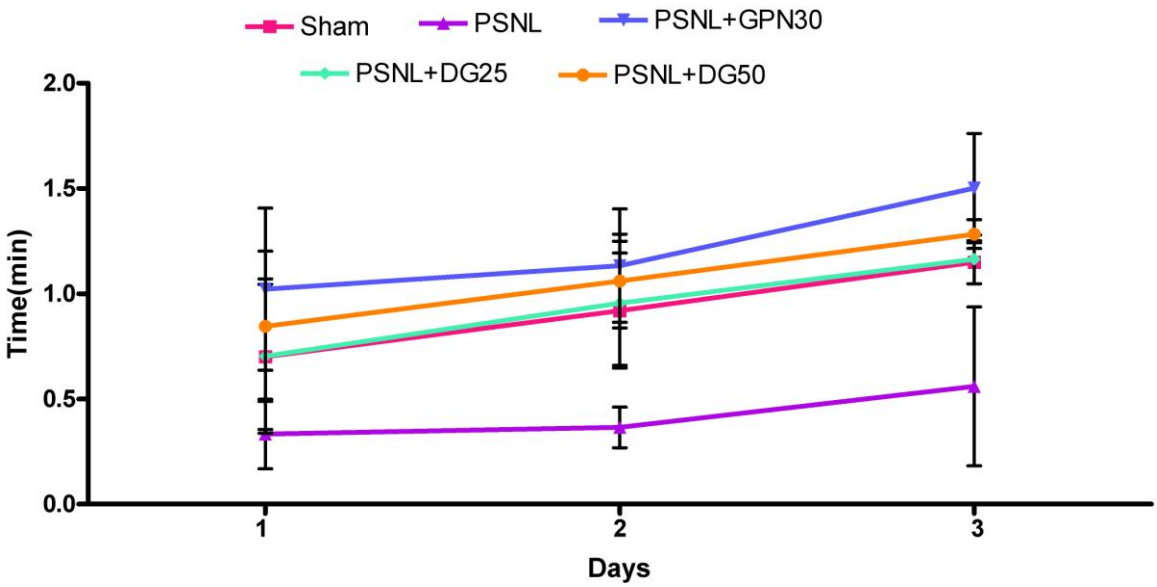
All the data were expressed as mean ± SD. One way ANOVA followed by post hoc tukey comparison test. PSNL=Partial sciatic nerve ligation GPN=Gabapentin DG=*Desmodium gangeticum* $^{\#\#}$**,** denotes statistical significance in comparison to sham treated group at $p<0.01$ respectively. *****,** denotes statistical significance in comparison to PSNL treated group at $p<0.001$ respectively.

NOVEL OBJECT RECOGNITION TEST

Time taken into initially enter the objects



Time spent in center zone



Time taken to initially enter and inspect objects

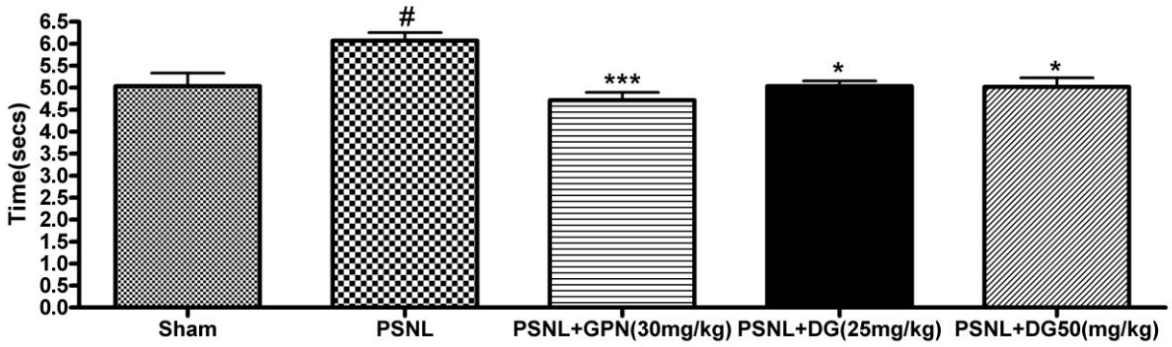
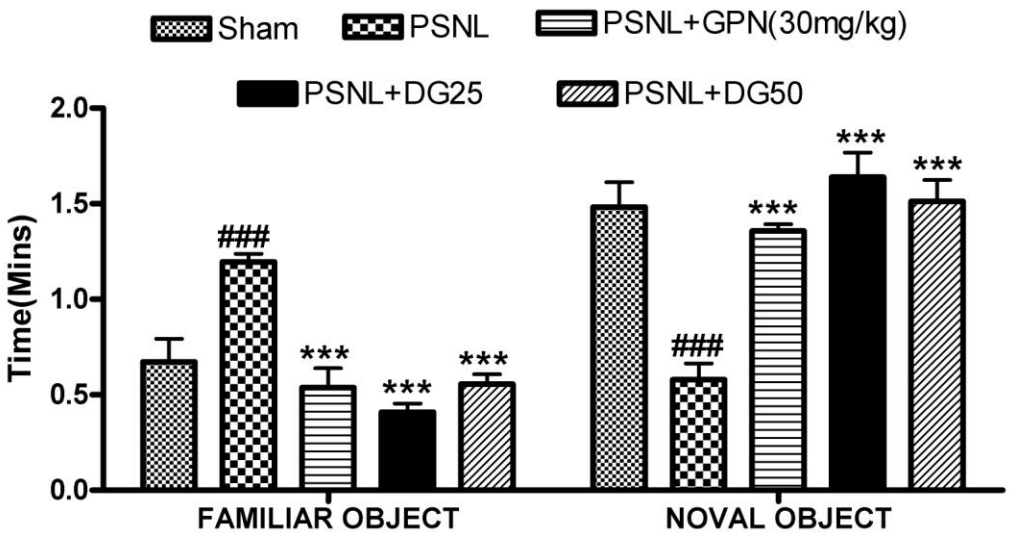


Figure 9.1: Effect of various treatments on novel object recognition test.

All the data were expressed as mean ± SD. One way ANOVA followed by post hoc tukey comparison test.PSNL=Partial sciatic nerve ligation GPN=Gabapentin DG=*Desmodium gangeticum* **#,** denotes statistical significance in comparison to sham treated group at $p<0.05$ respectively. *,*****,** denotes statistical significance in comparison to PSNL treated group at $p<0.01$,$p<0.001$ respectively .

Time taken inspecting each objects: (familiar object &novel object)



TIME SPENDING (%):-

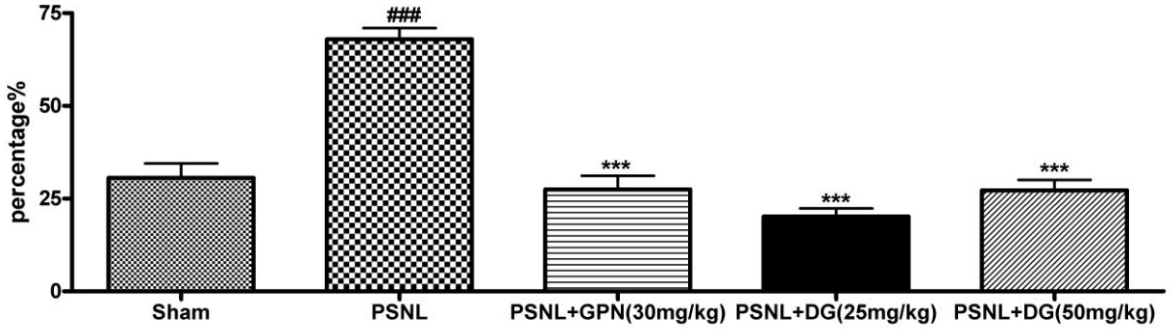
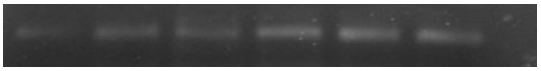
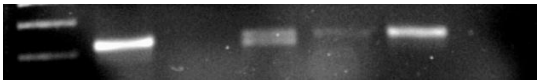


Figure 9.2: Effect of various treatments on novel object recognition test. All the data were expressed as mean ± SD. One way ANOVA followed by post hoc tukey comparison test.PSNL=Partial sciatic nerve ligation GPN=Gabapentin DG=*Desmodium gangeticum.* **###,** denotes statistical significance in comparison to sham treated group at p<0.001 respectively. *****,** denotes statistical significance in comparison to PSNL treated group at p<0.001 respectively.

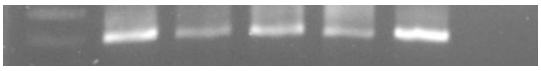
PCR STUDIES



cox



e-NOS



GAPDH

COX

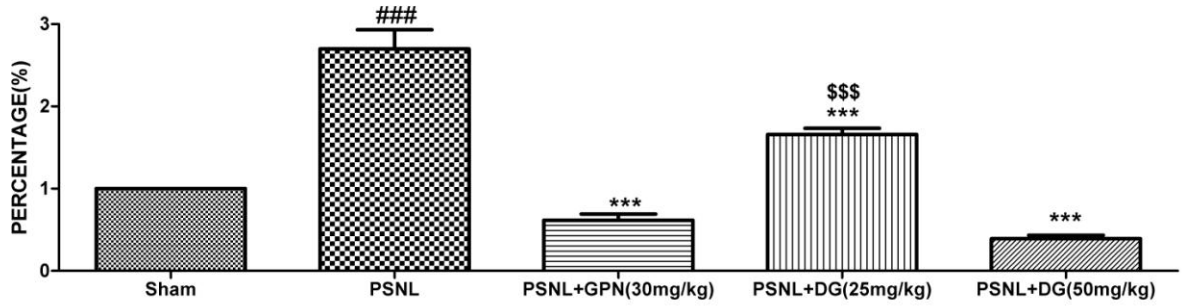


Figure 10.1: Quantification of cox mRNA level expression by conventional PCR .all the data were expressed as mean ± SD. One way ANOVA followed by post hoc tukey comparison test . PSNL=Partial sciatic nerve ligation GPN=Gabapentin DG=*Desmodium gangeticum*

###, denotes statistical significance in comparison to sham treated group at $p<0.001$ respectively.*****,** denotes statistical significance in comparison to PSNL treated group at $p<0.001$ respectively.$^{\$\$\$}$**,** denotes statistical significance in comparison to GPN treated group at $p<0.001$ respectively.

e-NOS

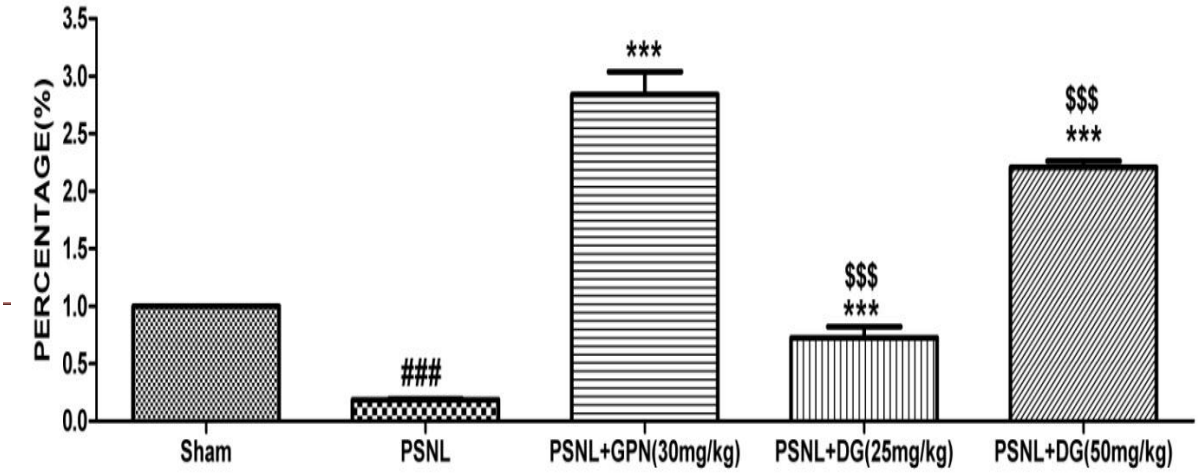
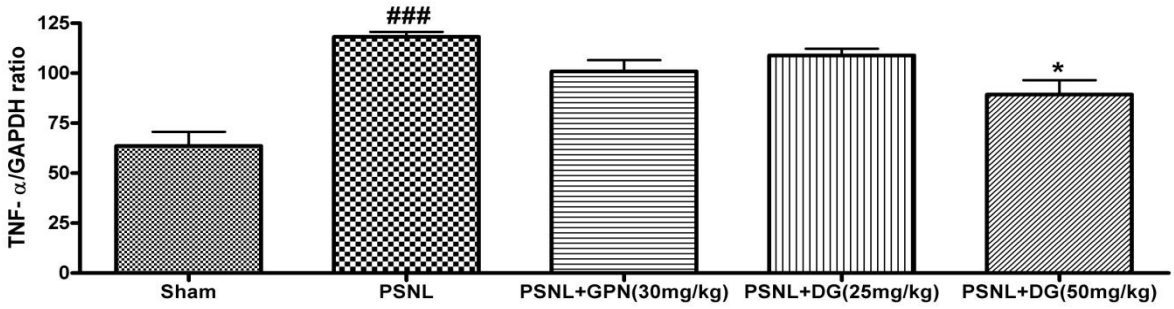


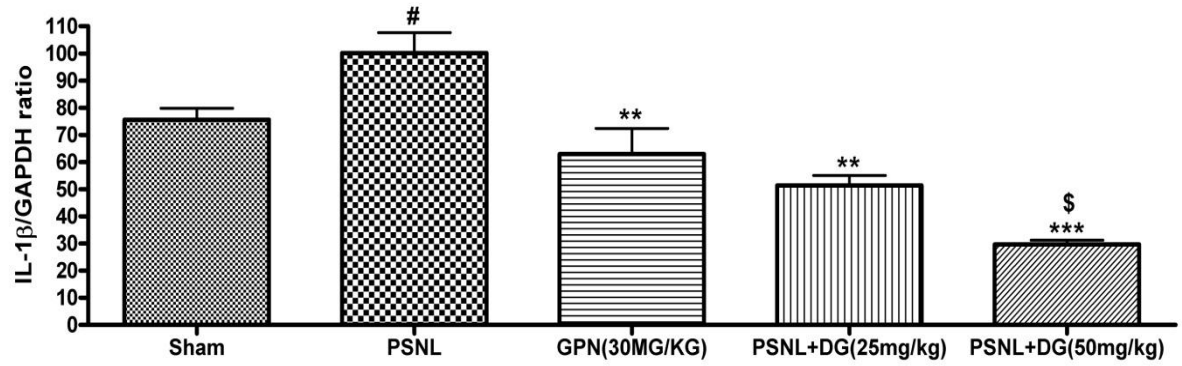
Figure 10.2: Quantification of e-NOS mRNA level expression by conventional PCR .All the data were expressed as mean ± SD. One way ANOVA followed by post hoc tukey comparison test . PSNL=Partial sciatic nerve ligation GPN=Gabapentin DG=*Desmodium gangeticum.* **###**, denotes statistical significance in comparison to sham treated group at p<0.001 respectively.*****,** denotes statistical significance in comparison to PSNL treated group at p<0.001 respectively. **$$$,** denotes statistical significance in comparison to GPN treated group at p<0.001 respectively.

ELISA TEST:-

TNF-α



IL-1β



IL-6

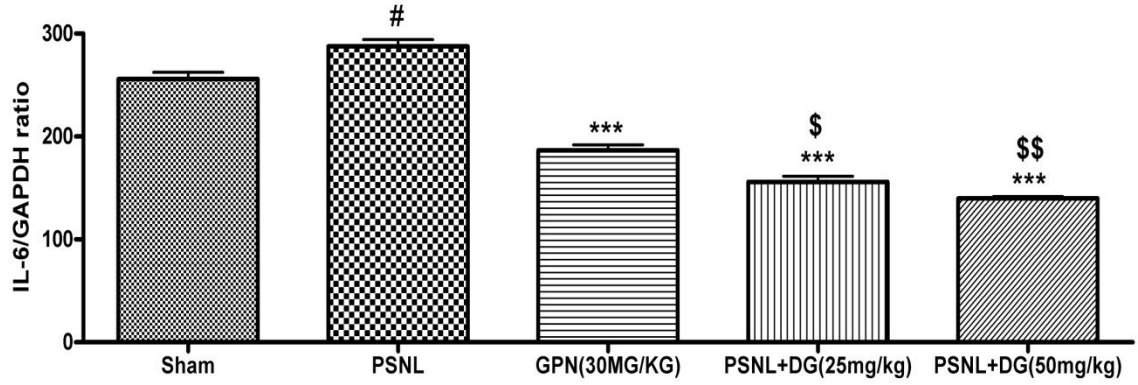
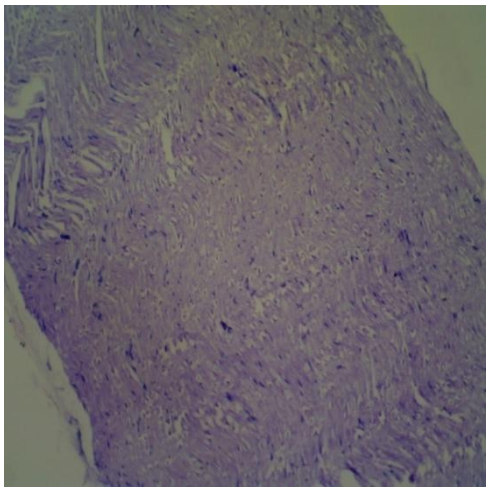


Figure 11: Quantification Of Tnf-α, Il-1β AND IL-6 Level By Elisa. All the data were expressed as mean ± SD. One way ANOVA followed by post hoc tukey comparison test. PSNL=Partial sciatic nerve ligation GPN=Gabapentin DG=*Desmodium gangeticum* $^{\#}$, denotes statistical significance in comparison to sham treated group at $p<0.05$ respectively.***, **,***** denotes statistical significance in comparison to PSNL treated group at $p<0.05$, $p<0.01$, $p<0.001$ respectively. $^{\$}$, $^{\$\$}$, denotes statistical significance in comparison to GPN treated group at $p<0.05$, $P<0.01$ respectively.

HISTOPATHOLOGY STUDIES

Sham Operated

Section studied from sciatic nerve shows normal axons, fibrocytes and schwann cells with focal degenerations noted. No evidence of inflammation/granuloma seen



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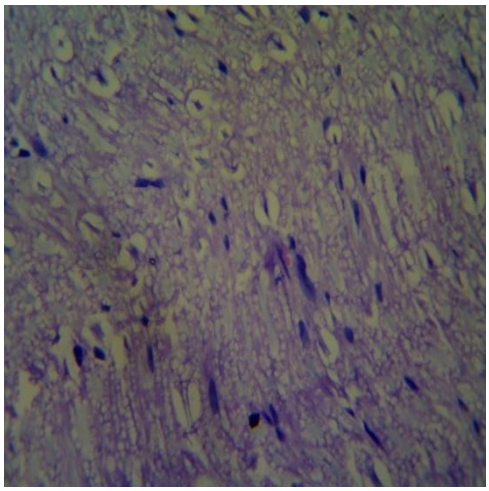

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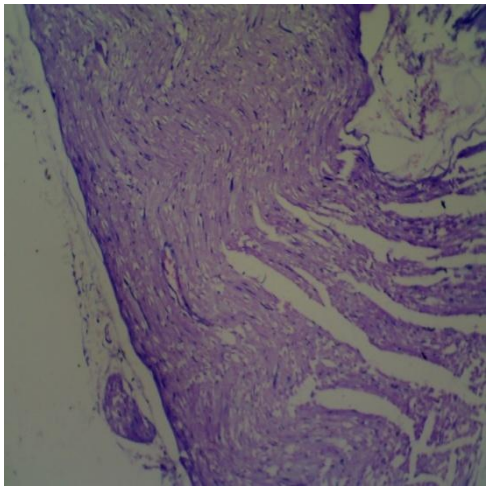
Figure 12.1: Microscopic observation

PSNL

Section studied from sciatic nerve shows normal axons, fibrocytes and schwann cells and nodes of ranvier with focal mild swelling of endoneurium.



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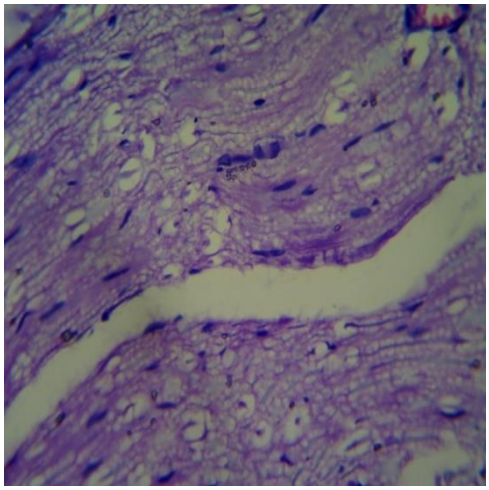
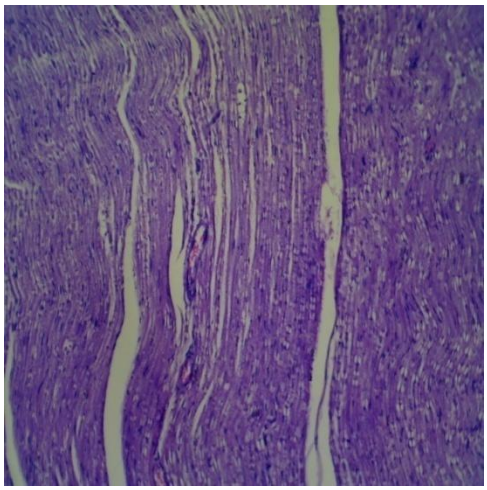


40X

Figure 12.2: MICROSCOPIC APPEARANCE

GABAPENTIN (30mg/kg)

Section studied from sciatic nerve .shows normal axons, fibrocytes and schwann cells, nodes of Ranvies, myelin sheeth and endoneurium. No inflammation/granuloma seen



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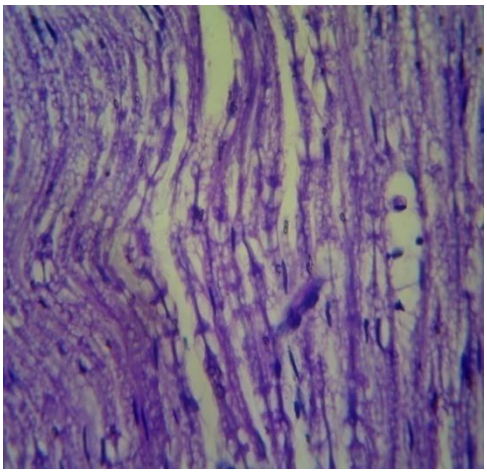
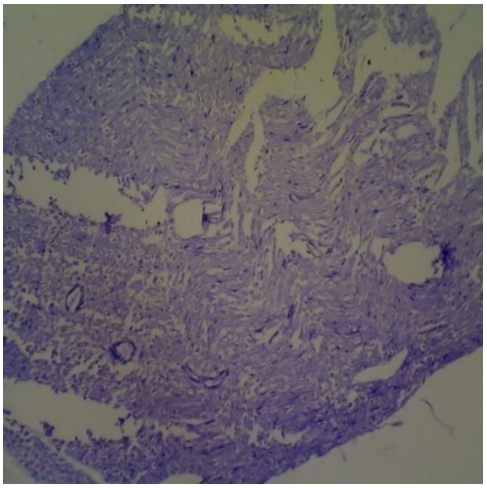


40x

Figure 12.3: MICROSCOPIC APPEARANCE

Desmodium gangeticum (25mg/kg)

Section studied from sciatic nerve shows normal axons, fibrocytes and schwann cells with focal degenerations noted.



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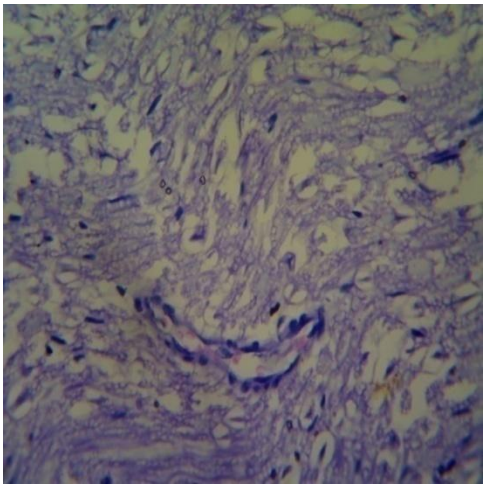
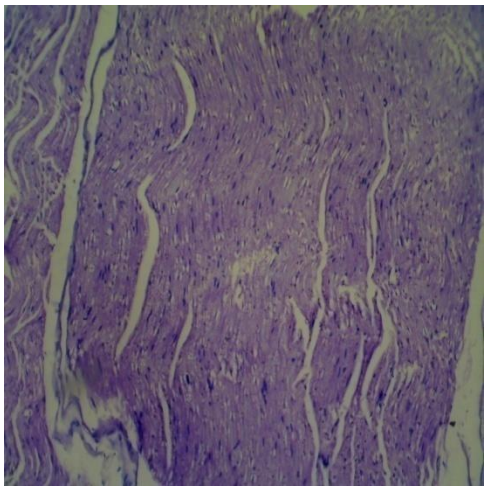


40X

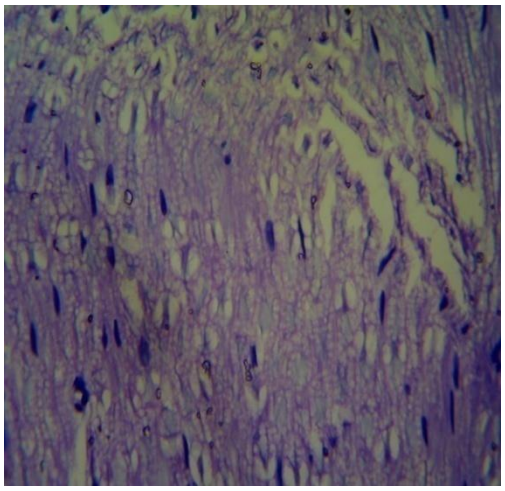
Figure 12.4: MICROSCOPIC APPEARANCE

Desmodium gangeticum (50mg/kg)

Section studied from sciatic nerve shows normal morphology. No evidence of degeneration/inflammation/granuloma.



10x



40x

Figure 12.5: MICROSCOPIC APPEARANCE

Chapter 6

Discussion

6. DISCUSSION

Neuropathic pain is caused by a lesion or dysfunction affecting the nervous systems. It is often manifested as spontaneous pain, hyperalgesia, and allodynia. The neuropathic pain is usually difficult to treat because the basic etiology is heterogeneous and the underlying pathophysiology is complex (Bhat *et al*., 2016). Growing evidence indicates that inflammatory and immune responses contribute to neuropathic pain. In addition, neuroinflammation induced by nervous system injury accompanies a neuroimmune interaction that activates immune cells and leads to development of neuropathic pain. *Desmodium gangeticum* (DG) has been reported to possess anti-inflammatory, hepatoprotective, antiamnesic, antinociceptive and wound healing activities (Ghosh & Anandkumar 1983; Prasad *et al.* 2005; Jain *et al.* 2006). The preliminary study on LPS stimulated RAW 264.7 cell lines revealed that *Desmodium gangeticum* (DG) posses anti-inflammatory activity *in vitro.*

Kim et al. reported that the partial sciatic nerve ligation(PSNL) neuropathic pain model produces significant responses to the hot-plate test, von Frey test and, supporting its usefulness and appropriateness in pain investigations for thermal hyperalgesia and mechanical allodynia. In our study, the results of the behavioral tests in PSNL operated rats have shown similar pain responses. The aqueous extract of *Desmodium gangeticum* attenuated the thermal hyperalgesia suggesting it may act via central through inhibitory nerve sensitivity. It has been reported that small unmyelinated and large myelinated primary nerves (Aβ and small-diameter nociceptive fibres) are implicated in mechanical allodynia (Field *et al.*, 1999). In our study, the aqueous extract of *Desmodium gangeticum* reversed the thermal allodynia induced by PSNL model. Mechanical hyperalgesia resulting from tissue injury or inflammation is often associated with sensitization of TRPV1 channel activity which can occur through several mechanisms such as

phosphorylation, interaction with phospholipid PIP2, trafficking, and association with accessory proteins. Many studies have implicated TRPV1 in the modulation of sensory transmission from the central terminals of primary afferents to spinal dorsal horn neurons (Kim *et al*., 2014; Choi *et al*., 2016). The aqueous extract of *Desmodium gangeticum* attenuated the hyperalgesia which suggest it may act through by inhibiting TRPV1 receptors.

Partial peripheral nerve injury leads to chronic pain states such as causalgia. The symptoms include spontaneous burning pain, thermal hyperalgesia and mechanical allodynia in the affected area which was also observed in our study. It is known that the axotomized sensory afferents produce abnormal spontaneous discharges (Han *et al*., 2000) leading to central sensitization, a state of increased excitability of spinal dorsal horn nociceptive cells following prolonged nociceptive input (Woolf, 1983; Woolf and Doubell, 1994). The aqueous extract of *Desmodium gangeticum* attenuated the allodynia and hyperalgesia induced by PSNL which suggest it may be useful in causalgia form pain disorders.

In our study, PSNL operated rats displayed significant mechanical allodynia, thermal and mechanical hyperalgesia, Neuropathic pain not only impacts negatively on physical and but psycho-logical functioning also. Impairments in cognitive domains including attention and working memory have been demonstrated in chronic pain patients using both subjective reports and objective neuropsychological testing (orla moriarty et al., 2016).In our study PSNL surgery affected spatial learning or memory in the traditional water-maze task. Hu et al., found impairments in a similar task in an L5-transection model of neuropathic pain. Further, treatment of aqueous extract of *Desmodium gangeticum* to PSNL rats reversed the cognitive impairment evaluated by morris water maze test.

In the novel-object recognition task, PSNL rats showed no preference for the novel object compared with the familiar object, and their exploration of the novel object was reduced compared with that of the sham operated group. This indicates a specific deficit in recognition memory and concordance with previous studies (orla moriarty et al., 2016). Previous studies have shown deficits in novel-object recognition associated with pain in sprague dawley rats in an inflammatory pain model (wen-jie-ren *et al.,* 2011) and in mouse neuropathic pain model. The treatment of aqueous extract of *Desmodium gangeticum* to PSNL rats improved the recognition memory suggesting it may alleviate pain associated memory impairment. In spared nerve injury (SNI) model of neuropathic pain, over-production of TNF-α following peripheral nerve injury has lead to neuropathic pain associated memory deficits and their inhibition of TNF-α or genetic deletion of TNF receptor 1 prevented both memory deficits and synaptic dysfunction induced by SNI (wen-jie-ren *et al.,* 2011).

Several studies have reported that pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin-1 (IL-1β & IL-6) and nuclear factor-kappa B (NF-κB) may contribute to pathogenesis of neuropathic pain, with peripheral and or central nervous system mechanisms. Pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor TNF-α are produced after nerve injury and play an essential role in neuropathic pain sensitization (Strichartz *et al.,* 2004, Cheng *et al.,* 2008a). IL- 1β and TNF- α play an important role in the amplification loop of the nociceptive response as they can stimulate their own and each other's production (Dinarello *et al.,* 1997). The activated microglia mainly produces these toxic cytokines IL-1, IL-6, & TNF- α as well as nitric oxide, excitatory amino acids, ATP, and prostaglandins in neuropathic pain (Inoue, 2006). Our study has shown that aqueous extract of *Desmodium gangeticum* down regulated TNFα and IL1β at nerve levels in PSNL pain model.

The aqueous extract of *Desmodium gangeticum* also down regulated the level of IL-6 in PSNL pain model.

Inflammatory cytokines induce COX-2 in which manifests inflammatory pain via PG in various cells and selective inhibitors of COX-2 block pain in inflammatory models and are studied in clinical trials (Shigeru Kobayash *et al.,* 2015). The COX-2 expressing cell profiles were present in 2^{nd} and 4^{th} week injured sciatic nerve following partial sciatic nerve ligation. Ma et al. demonstrated that partial nerve injury induces more abundant COX-2 expression than complete nerve injury (Talmar *et al.,* 2003). In the study, PSNL operated rats had elevated cox-2 levels and their expression was down regulated by treatment of aqueous extract of *Desmodium gangeticum.*

eNOS is mainly produced by endothelial cells and may be produced by Schwann cells (Kobayashi *et al.,* 2009). WEN-NING QI, *et al.,* 2001 studied the expression of different nitric oxide synthases in ischemia-reperfused peripheral nerve of the rat. In his study, after 3 h of reperfusion, expression of eNOS mRNA and protein was down regulated, that lead to neuronal cell damage. In our study, PSNL operated rats had reduced eNOS mRNA levels and treatment of aqueous extract of *Desmodium gangeticum. p*were unregulated their mRNA levels in PSNL injured rats.

Chapter 7

Summary & Conclusion

7. SUMMARY & CONCLUSION

- The present study aimed to evaluate the aqueous extract of *Desmodium gangeticum* for its protective effect in neuropathic pain activity by using *in vitro* and *in vivo* models like LPS induced inflammation in murine macrophages and PSNL model to induce neuropathic pain in rats.
- We observed the pain and memory functions condition of animals by inducing neuropathic pain using PSNL model in rats. To elucidate the mechanism of action protective effect in neuropathic pain, pro-inflammatory mediators were measured. To support these studies histopathological studies is carried out.
- PSNL rats showed that significant decrease in pain threshold in pain behaviour studies such as thermal, mechanical allodynia and hyperalgesia and reduced motor coordination and cognitive impairment functions.
- Aqueous extract of *Desmodium gangeticum* was prepared by water decoction process. Quantification through HPTLC studies revealed that *Desmodium gangeticum* contained 0.608mg/g amounts of Quercetin.
- *Desmodium gangeticum* exhibited dose dependent protection in cell viability in LPS induced inflammation in murine macrophages.
- The aqueous extract of *Desmodium gangeticum* show significant increase in pain threshold in pain behaviour studies such as thermal, mechanical allodynia and hyperalgesia.
- The aqueous extract of *Desmodium gangeticum* improved motor coordination and cognitive impairment.
- The aqueous extract of DG showed significant decrease in pro-inflammatory mediators like TNF-α, IL- 1β, IL-6, COX-2 which shows that anti-inflammatory mechanism of *Desmodium gangeticum.*

The study can be concluded that treatment of *Desmodium gangeticum* in PSNL induced rat reduced mechanical and thermal hyperalgesia and allodynia. It also improved the long term spatial memory and recognition memory. They exert their activity by inhibiting pro inflammatory mediator such as TNF-α, IL- 1β, IL-6, and COX-2 in PSNL affected nerves. The outcome of the study is that the aqueous extract of *Desmodium gangeticum* may be a potential therapeutic usage as novel anti nociceptive and cognitive impairment therapy in chronic neuropathic condition.

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Annexure

भारत सरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA
दक्षिणी क्षेत्रीय केन्द्र / Southern Regional Centre
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सं. भा.व.स./द.क्षे.के./No.: BSIS/RC/5/23/2016/Tech./2074 दिनांक/Date: 21st December 2016

सेवा में / To

Mr. Vijaya Ragavan. A
M. Pharm. Pharmacology
Department of Pharmacology
PSG College of Pharmacy
Coimbatore – 641 004

महोदय/Sir,

The plant specimen brought by you for identification is identified as *Desmodium gangeticum* (L.) DC. (= *Hedysarum gangeticum* L.) – LEGUMINOSAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

धन्यवाद/Thanking you,

भवदीय/Yours faithfully,

GVSMurthy 21/12/16

(डॉ. जी.वी.एस. मूर्ति /Dr. G.V.S. Murthy)
वैज्ञानिक 'जी' एवं कार्यालय अध्यक्ष /
Scientist 'G' & Head of Office

वैज्ञानिक 'जी' एवंकार्यालयअध्यक्ष
SCIENTIST 'G' & Head of Office
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PSG Institute of Medical Sciences & Research
Institutional Animal Ethics Committee

Registration No. : 158 / PO / ReBi / SL / 99 / CPCSEA
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Phone : 91 422 - 2570170, 2598822 Fax : 91 422 - 2594400 Email : psganimalethics@gmail.com

DATE: 22.09.2016

Title of the Project: Neuro pharmacological assessment of Desmodium *gangeticum* in partial Sciatic Nerve ligation rat model.

Proposal Number: 335 /2016/ IAEC

Name of the Applicant: Vijaya Ragavan.A

Approval date: 22.09.2016

Expiry date (Termination of the Project): 21.09.2017

Methodology: Approved.

Name of species: **Swiss albino mice/ Wistar rats/ Sprague Dawley rats/ Guinea pigs/ Newzealand White rabbits.**

Male/Female/Both sex----24----animals approved.

Signature of Chairperson

Date: 22.09.16

Dr.M.Ramanathan

Name of the chairperson

The Chair Person, CPCSEA
IAEC of PSGIMS&R
Coimbatore-641 004.

Signature of the CPCSEA nominee

Date: 22/09/2016

Dr.C.Kathirvelan

Name of IAEC/CPCSEA nominee

Main Nominee, CPCSEA
IAEC of PSGIMS&R
Coimbatore-641 004.

49th Annual Conference of
Indian Pharmacological Society
IPSCON 2016
20-23 October 2016
Postgraduate Institute of Medical Education and Research, Chandigarh

"New Paradigms for Advancing Therapeutic Horizon in Pharmacology for Global Health:
Challenges for Drug Development, Regulation, Future of Pharmacology & Therapeutics and to Educate Next Generation of Pharmacologist"

Launching Golden Jubilee Year of IPS

Certificate

This certificate is awarded to

Prof./Dr./Mr./Ms. A. Vijayaraghavan

for his/her presence in the

49th Annual Conference of Indian Pharmacological Society

As a Participant

AT

POSTGRADUATE INSTITUTE OF MEDICAL EDUCATION AND RESEARCH

Chandigarh on 20-23rd October 2016

Punjab Medical Council has granted 11 credit hours for this program vide

Letter No. PMC/CME/2016/13194 dated 18.08.2016

Dr. B. Dinesh Kumar
President
Indian Pharmacological Society

Prof. Bikash Medhi
Organizing Secretary
IPSCON 2016, PGIMER, Chandigarh

Dr. Manjit Kaur Mohi
Registrar
Punjab Medical Council

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH

COIMBATORE - 641 004

CME Accreditation Certificate

This is to certify that Vijay Ragavan

has participated in

MOLECULAR BASIS OF MALIGNANCY - CURRENT TRENDS

held at PSG IMS&R on 23-06-16 as a participant

This activity has been reviewed and accepted by The Centre for Accreditation, The Tamil Nadu Dr. MGR Medical University and the University designates this educational activity for a maximum of 05 Credit points in Category II

Moderator

Dr. S. Ramalingam
Dean